universität wien

# DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„Combinarory endocrine activity of mycoestrogens and phytoestrogens“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Doktor der Naturwissenschaften (Dr. rer. nat)

Wien, 2023 / Vienna 2023

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| Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet: | UA 796 605 419 |
| Dissertationsgebiet lt. Studienblatt / field of study as it appears on the student record sheet: | Doktoratsstudium Naturwissenschaften: Chemie |
| Betreut von / Supervisor: | Univ.-Prof. Dr. Doris Marko |
| Mitbetreut von / Co-Supervisor: | Dr. Elisabeth Varga |

# Acknowledgements

I am very grateful, and I would like to express my deepest appreciation to a number of people, who have been instrumental in getting this thesis to a successful completion.

I would like to thank my supervisor Doris Marko for giving me the opportunity to be part of this inspiring and instructive project. Furthermore, I am thankful for her meaningful assistance, her trust and supportive guidance.

Furthermore, I specially would like to thank Dr. Elisabeth Varga for her fantastic supervision. I could not have wished for a better supervisor. I am extremely grateful for all the great support you offered me during the whole time and for answering all my questions. Additionally, I am thankful for your confidence in me. I learned so much, scientifically and personally during the last years.

I would like to express my sincere gratitude to my cooperation partner, Barbara Novak, from dsm - firmenich for her invaluable contributions and unwavering commitment throughout our collaboration. Barbara's dedication, expertise, and collaborative spirit were instrumental in the success of this project.

I would like to express my gratitude to all the members of the working group. Each of us has his or her crazy issues – thanks for accepting me and my craziness as I am. You helped me through every single phase of this PhD thesis and I cannot remember how often I cried with you for joy. It was my pleasure working and being with you.

Thank you to all my friends for finding the right balance between supporting me when I urgently needed them by my side and giving me the time and showing understanding when I spent hours, days and nights working.

Finally, I would like to thank my whole family – the Grgics and the Pavlovics. Especially, my parents for never-ending support, trust and acceptance. My sister, her husband and my two nieces for honesty and simply being who they are.

…THANK YOU..

# Contents

# 1. Introduction

Today's commercially available pig feed usually consists of several components: a high-protein source, carbohydrate-rich grains, vitamins, and minerals [1]. Due to an increased global production and a high protein content of 38 %, soybeans are the most popular protein source for pig feed [2]. With a worldwide annual production of over 1 billion tons [3] and the fact that most of the nutrients are easily digestible by pigs [4] corn is the most popular source of carbohydrates[1]. However, according to the latest data, up to 80 % of the grain is contaminated with mycotoxins [5]. The global contamination of feed with mycotoxins (toxic secondary metabolites of molds) presents a significant problem and is affected by several factors that influence the presence of these compounds in feed. While the storage of products can be easily controlled, other environmental factors such as the climate are more difficult to influence and have to be checked accordingly [6]. The resulting exposure to these secondary metabolites can negatively impact people, animals, and plants, leading to illnesses and economic losses [6].

In case of corn, mycotoxins were detected in over 90 % of the tested samples, with deoxynivalenol (DON, 72 %) and zearalenone (ZEN, 49 %) being the most frequently occurring toxins [7]. A large number of different species are accountable for the production of mycotoxins and fungal diseases. Mycotoxins produced by these fungi vary in their chemical structure and properties that are responsible for their different biological and toxicological effects [8].

One of the most common mycotoxins found in corn is the previously mentioned Fusarium mycotoxin zearalenone (ZEN). Since June 2005, the EU has implemented regulatory limits for the allowable amount of ZEN in grain. For corn, the maximum permissible ZEN value in ready to eat sow feed is 200 µg/kg [9,10]. However, this value is often exceeded in many corn samples [5,7,11].

ZEN and its derivatives belong to the group of macrocyclic resorcylic acid lactones [12]. The toxic effects of ZEN are extensive. Alongside documented *in vitro* effects such as genotoxicity, carcinogenicity, and immunotoxicity (chapter 2.1.6), ZEN is most notable for its estrogenic potential (chapter 2.1.8) [13,14].

In addition to the mycoestrogen ZEN, plant constituents may also possess estrogenic potential. One example is isoflavones (ISF) found in soy, which are diphenolic molecules classified as phytoestrogens [15]. The observation of the "clover disease" in sheep in 1940 due to excessive consumption of ISF from subterranean clover marked the first instance of this effect. The reproductive system of the animals was primarily affected, resulting in symptoms such as estrus disruption, increased endometriosis, infertility, and abortions within the majority of the herd [16].

According to the US Environmental Protection Agency (US EPA), endocrine disrupting chemicals (EDCs) are exogenous agents that interfere with the synthesis, metabolism and action of endogenous hormones [17]. The main way EDC influences the endocrine system is based on the structural similarity with 17β-estradiol (E2), which leads to interactions with hormone receptors, especially concerning the reproductive system. In pigs, exposure to EDC can lead to hyperestrogenism, affecting sexual maturation, estrus and reproduction [18–21]. The possible mode of action of the estrogenic effects of ZEN and ISF is discussed in chapter 2.4.

As both mycoestrogens and phytoestrogens coexist in feed, it is reasonable to assume that interactions between these two substance classes occur, potentially leading to synergistic or antagonistic estrogenic effects. For instance, Vejdovszky *et al.* demonstrated an interactive effect between ZEN and genistein (GEN) concerning estrogenic properties *in vitro*. Depending on the ratios and concentrations used, a synergistic to a strong synergistic estrogenic effect could be induced by the combination of these two substances [22]. Nevertheless, data on the combined effects of phyto- and mycoestrogens remains limited.

Potential interactions among xenoestrogens warrant a reassessment of the risk posed by endocrine-disrupting substances. To enhance the understanding of the effects of phytoestrogens and mycoestrogens, this dissertation aims to integrate current knowledge regarding the occurrence and impacts of ISF and the mycoestrogen ZEN, as well as its metabolites, on farm animals.

# 2. Objectives of the thesis

The principal aim of this thesis is to delve into the intricate consequences arising from the simultaneous presence of mycoestrogens, bioactive metabolites generated by specific fungi with the capability to disrupt endocrine functions, and phytoestrogens, organic compounds derived from plants that hold estrogen-like and anti-estrogenic attributes. Our focus is centered on unraveling the intricate interplay wherein phytoestrogens might influence the impacts of mycoestrogens on hormone-responsive cells. This inquiry leverages advanced *in vitro* cellular models, placing significant emphasis on two cell lines: the Ishikawa cell line, representative of human endometrial adenocarcinoma bearing both isoforms of the estrogen receptor, and the hERα-HeLa-9903 cell line, a variant of human cervix cancer cells engineered to express the estrogen receptor ERα. Through these models, our goal is to gauge the estrogenic potential and delve into estrogen receptor activation.

To assess that, the main goal of this thesis is to study the estrogenic activity of binary combinations of mycoestrogens and phytoestrogens, both in isolated form and when co-occurring, using hormone-sensitive cell models. The Ishikawa cell model was used to quantitatively measure the estrogenic potential of individual substances by monitoring alkaline phosphatase (ALP) activity, which reflects estrogen receptor activation. Furthermore, the hERα-HeLa-9903 cell line using the luciferase reporter assay was utilized to evaluate the estrogenic response. Based on the ALP measurements in the Ishikawa cell model, selected combinations using the hERα-HeLa-9903 cell model expressing solely the ERα were tested. It was hypothesized that the enhanced estrogenic effects of combinations between phyto- and mycoestrogens are based on the interaction of the respective substances with both ERs. While ZEN and its metabolites have a relative high affinity to both ERs, ISF preferably interact with ER β [23–25]. Therefore, we did not expect enhanced estrogenic effects in the hERα-HeLa-9903 cell line.

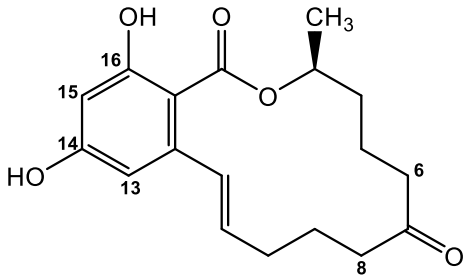
The goal is to provide a deeper understanding of the interactions occurring between myco- and phytoestrogens, thereby contributing to the collective knowledge of their combined effects on hormone-sensitive cells. However, risk assessments are still predominantly based on the toxicological data of single substances, which often underestimate the toxicological potential of certain mixtures. Data on combinatorial effects are still scarce and therefore, this thesis should provide a deeper insight in combinatorial toxicological effects.

# 3. Theoretical background

## 3.1 Zearalenone

### 3.1.1 Chemical and physical data of zearalenone

Zearalenone (ZEN), also known as 6-[10S-hydroxy-6-oxo-trans-1-undecenyl]-β-resorcylic acid lactone, is a crystalline, white compound with a melting point of 164-165 °C and a molecular weight of 318 g/mol. It is soluble in organic solvents such as methanol, dimethylsulfoxide (DMSO) and chloroform, as well as in weak alkaline solutions. ZEN consists of two rings, one derived from resorcylic acid and the other from a lactone framework, connected by an intramolecular ester bond [26].



Zearalenone (ZEN)

Figure 1: Structure of zearalenone (ZEN)

### 3.1.2 Natural occurrence and levels of zearalenone in food and feed

*Fusarium* thrives in the upper layers of the soil and decomposes cellulose-containing plant residues. It prefers high humidity and moderate to warm climates. *Fusarium* growth is influenced by moisture content, temperature, and humidity of cereal grains. Factors such as mild winters, rainy summer months, insect infestation, and delayed harvesting can contribute to increased mycotoxin levels. Preventive measures include maintaining low humidity during grain storage, periodic soil cultivation to bury plant residues, and crop rotation [27]. The main producers of ZEN are *Fusarium graminearum*, *Fusarium moniliforme*, and *Fusarium avenaceum*, although other *Fusarium* species can also synthesize this mycotoxin. These fungi are commonly found in grains, particularly corn, barley, and wheat, with infestation occurring before harvest, mainly during the flowering period [28].

ZEN can be emitted from *Fusarium*-infected fields through drainage water. Critical levels of ZEN can be found primarily in small rivers next to cultivated fields, leading to common contamination of ZEN and deoxynivalenol (DON) [29].

Grains, especially corn, are frequently contaminated with ZEN, and other food and feed items derived from contaminated grains, such as nuts, crackers, chips, cereal, and infant formula, can also be affected. ZEN levels in various food and feed typically range from micrograms to milligrams per kilogram of food [7].

Figure 2: Corn hanging up in the shed (https://commons.wikimedia.org/wiki/Category:Maize#/media/File:Corn_hanging_up_in_the_shed_above_the_house_Nanyao_Village,_Naxi,_Yunnan.jpg)

### 3.1.4 Regulatory limits and daily intake of zearalenone

The maximum total amount of ZEN within the EU is strongly regulated for various food and feed. Corn and corn products for direct human consumption have the highest limit of 200 µg/kg, while cereal-based complementary foods for infants and young children have a limit of one tenth of this value. For feed designated for sows the total amount of ZEN is 200 µg/kg, whereas for piglets the value is 100 µg/kg [9,26,30].

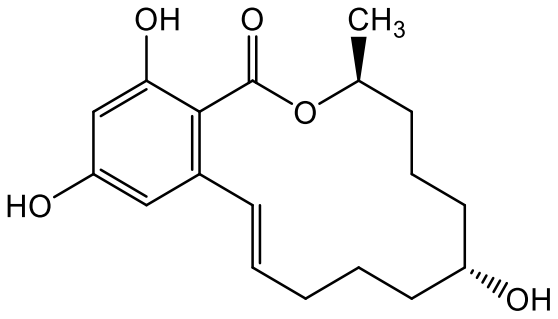
The daily intake of ZEN depends on an individual's diet and is estimated to range from 0.03 to 0.06 µg/kg body weight. The intake primarily occurs through bread and grain products made from wheat, rye, oats, and corn. A carry-over effect on milk, eggs, and meat from livestock has been demonstrated, however in non-relevant concentrations regarding possible toxic effects. Despite

this, the estimated average intake of ZEN remains below the maximum tolerable daily intake of 0.25 µg/kg bw/day [9,26].

### 3.1.5. Metabolism

ZEN is easily absorbed by the body due to its lipophilic nature. The distribution coefficient of ZEN in the octanol-water system, known as the Pow value, reflects its polarity. The logarithmic Pow value of ZEN is 3.2, indicating its rapid absorption after oral intake in rats, rabbits, pigs and humans. In pigs, the bioavailability of ZEN ranges from 80 % to 85 % [11,31,32]. However, in rats, the absolute bioavailability of ZEN is low due to extensive pre-systemic metabolism. Significant differences in biotransformation and metabolic profiles are found between animal species [26,33,34]. The intestine serves as the initial protective barrier against ZEN in two ways. It can limit the passive movement of ZEN through the cell membrane due to its lipophilic nature, and actively transport intracellularly diffused ZEN molecules back into the intestinal lumen using transporter proteins. Additionally, the intestine is a site where ZEN undergoes metabolism, forming various metabolites. This makes it important to study how ZEN is absorbed and metabolized in this barrier, to estimate the actual amount that gets absorbed into the body. Some studies have used human Caco-2 cells to examine these processes, finding that ZEN is quickly transformed into its metabolites in the intestine [35].

Once absorbed, ZEN can be transported in the body by binding to albumin or sex hormone-binding globulin (SHBG) after entering the portal blood. In the liver, ZEN undergoes conjugation (phase I and II metabolism) and is subsequently excreted via bile (feces) and kidneys (urine) [36]. The phase I metabolism involves enzymatic reduction catalyzed by 3α- and 3β-hydroxysteroid dehydrogenase, leading to the formation of α-zearalenol (α-ZEL) (higher estrogenic affinity than ZEN) and β-ZEL (lower estrogenic affinity). Pigs primarily produce α-ZEL, explaining their vulnerability to the exposure of ZEN. Different species and intraspecies metabolism of ZEN and its metabolites result in varying estrogenic potencies [26,33,34,37].



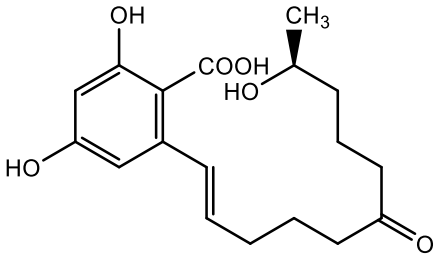

α-Zearalenol (α-ZEL)

Figure 3: Chemical structure of *α*-ZEL

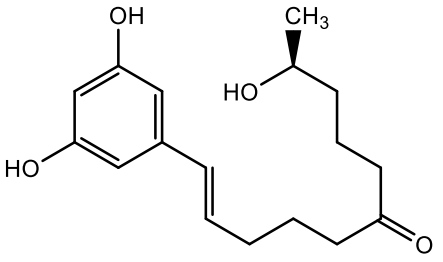
In phase II metabolism, ZEN and its metabolites are conjugated with glucuronic acid, and sulfates, predominantly in the liver and intestine. ZEN conjugates can be hydrolyzed by human colonic flora, restoring their estrogenic effects. Limited data exist on the *in vivo* absorption, bioavailability, and metabolism of phase I and phase II metabolites of ZEN [38,39].

Monohydroxylation of ZEN occurs at various positions by human cytochromes P450 and human liver microsomes. Aliphatic C6/8-hydroxy-ZEN appears less estrogenic than ZEN, while the toxicological relevance of aromatic metabolism remains poorly understood [40].

ZEN-sulfates, like ZEN-14-sulfate, are present as phase II metabolites in small amounts in animals and are also produced by certain fungi [41]. Chemical and physical detoxification methods result in unknown metabolites and nutrient losses, while ZEN degrading enzymes, such as those found in *Trichosporon mycotoxinivorans*, can form hydrolyzed ZEN (HZEN) by cleaving the lactone. HZEN and its decarboxylated form (DHZEN) are non-estrogenic and considered promising detoxification measures, making them promising candidates for mitigating the harmful effects of ZEN [42–44].




Hydrolyzed zearalenone (HZEN)




Decarboxylated hydrolyzed zearalenone (DHZEN)

Figure 4: Chemical structure of HZEN (left) and DHZEN (right)

*3.1.5.1 Metabolism in pigs*

An *in vitro* study of the metabolism of ZEN in the S-9 liver fractions from different species, showed that mainly the reductive metabolites α- and β-ZEL are produced by these fractions [45]. In animals, the conversion of ZEN to α- and β-ZEL depends on pH, tissue and sex. In pigs, ZEN is reduced to the aforementioned metabolites in the liver and mucosal cells of the duodeum and jejunum in the presence of NADPH [46]. The conversion process also involves the enzyme 3-α- and β-hydroxysteroid dehydrogenase (HSD) [47]. Intestinal homogenate shows only low levels of metabolic activity compared to the liver. However, the large intestinal surface area must also be taken into account, which in turn may contribute to a considerable amount of metabolic conversion [46].

*In vivo* studies investigating the urine and feces of pigs exposed to ZEN confirm *in vitro* experiments and show high concentrations of α- and β-ZEL in the feces after ZEN exposure [48]. The higher affinity of α-ZEL to the estrogen receptor compared to ZEN is due to the reduction of the 6-keto and vinyl group and the fact that α-ZEL does not bind to carrier proteins. This may lead to increased concentrations in serum [13,49].

Studies on ZEN metabolism showed that after oral administration of 90 mg ZEN per kg body weight, ZEN can be detected in plasma after one hour. After another hour, the main metabolite α-ZEL is also detectable in plasma. The maximum concentrations of ZEN and α-ZEL in plasma were determined 4 and 7 hours after ZEN administration, respectively. After enzymatic treatment with the enzyme glucuronidase, both ZEN and α-ZEL concentrations increased significantly [50]. This fact again suggests that ZEN is glucuronidated in the phase II metabolism and excreted via urine. Other experiments on ZEN metabolism indicated that mainly free ZEN is excreted in the urine [48]. These different results may be due to the different age, size, and timing of the measurements.

In addition to α- and β-ZEL, other metabolites were detected in pigs after ZEN administration. Zearalanone (ZAN) is a metabolite of zearalenone that is formed through the reduction of the keto group on the molecule. This means that a hydrogen atom is added to the keto functional group, resulting in a reduction in its overall chemical reactivity. Zearalanol (ZAL) is another metabolite of zearalenone that can be formed through further reduction of zearalanone. Like zearalanone, zearalanol also retains some estrogenic activity, though it is even less potent than both zearalenone and zearalenone, but these were detected only in very small amounts [51].

Dänicke *et al.* were able to demonstrate in an experiment that ZEN is subject to enterohepatic circulation. In this study, bile was collected from ZEN-exposed animals over half a day and was subsequently administered to animals that were not fed with ZEN. In the originally untreated

animals, ZEN could now be detected in the blood, demonstrating that ZEN is excreted via the bile and reabsorbed via the intestine [52]. This findings were also observed in piglets [31]. Furthermore, phase II metabolites of ZEN and its reductive metabolites are also detectable in the urine and feces of pigs, these being mainly glucuronide and sulfate metabolites [48,51].
The metabolic pattern formed in pigs appears to be dependent on factors such as age and body weight. In adult pigs, primarily ZEN metabolites are determined in the different tissues [51,53,54]. Whereas in recently born and young pigs, primarily ZEN is detected [48,50,55]. These differences in metabolic pattern can be explained by the unequal activity of the enzyme HSD [56].

### 3.1.6 Carcinogenicity, mutagenicity and genotoxicity

Through research conducted on mice it has been discovered that higher doses of ZEN can lead to the development of carcinomas, in the gland and liver tumors. However, when rats were exposed to doses ranging from 1 to 3 mg ZEN per kilogram of body weight, no increase, in tumor incidence was observed [36]. The potential carcinogenic effects of ZEN is attributed to its estrogenic activity, which is believed to affect cell proliferation and differentiation by interacting with estrogen receptors [13].
Various studies conducted on Vero cells and Chinese hamster ovary cells have revealed that ZEN has the potential to cause abnormalities in chromosomes exchanges, between sister chromatids and polyploidy. These findings suggest that ZEN possesses properties and can lead to an increase in damage [57]. Moreover, ZEN has been observed to induce mutations [36]. The genotoxic effects of ZEN are thought to be linked to its metabolites α-ZEL and β-ZEL [36].
*In vitro* experiments using cultured cells have demonstrated the genotoxic effects of ZEN, as it can induce DNA damage and formation of DNA adducts [58]. Studies in mice have shown the formation of DNA adducts in the liver and kidney, with a higher degree of adduct formation in the liver [59]. Notably, the formation of DNA adducts was more pronounced after intraperitoneal administration compared to oral administration, highlighting the importance of route-specific effects, albeit higher concentrations were used [60]. Rats, however, did not exhibit DNA adduct formation at lower doses of ZEN, suggesting potential species differences in genotoxicity [26].

### 3.1.7 Immunotoxicity

*In vitro* studies have shown that when ZEN is present, it can cause alterations in the immune system. For instance, it has been observed to inhibit the proliferation of lymphocytes stimulated by substances. Furthermore, ZEN also led to an increased production of specific cytokines [36]. These effects on the system are believed to be a result of ZENs ability to interact with estrogen

receptors, thereby influencing their function and response [61]. However, other studies did not observe any changes in immune parameters. This suggests that there might be species and concentration dependency concerning the immunotoxicity caused by ZEN [36].

### 3.1.8 Estrogenicity

The way a substance affects estrogenic stimuli is determined by how strongly it attaches to estrogen receptors (ERs), specifically ERα and ERβ. These receptors are part of a group called nuclear receptor subfamily 3, group I (NR3I), and they are found in various tissues [62]. When an estrogenic substance enters a cell, it interacts with receptor molecules, translocates within the activated receptor complex into the nucleus, and binds to specific areas on DNA known as estrogen response elements. This interaction either turns on genes with the help of coactivators or turns them off using corepressors [62].

ZEN behaves as both a ligand for ERα and a partial ligand for ERβ. This influences the genes controlled by these receptors. The estrogenic potential of ZEN and several of its metabolites is as follows: α-ZAL > α-ZEL > β-ZAL > ZEN > β-ZEL. In experiments on young mice, ZEN's estrogenic power is about 1/1000th of that of E2, while α-ZEL is seven times more effective than ZEN. Metabolization to α-ZEL increases its estrogenic impact, whereas β-ZEL decreases its impact [63].

ZEN's estrogenic effect has important implications for reproductive well-being. Increased levels of natural estrogen like E2 can hinder egg maturation and disrupt chromosome arrangements during egg cell division. ZEN shows similar effects, causing abnormal chromosomes and disturbing structures called microtubules in pig egg cells. Various animal studies have proven that ZEN exposure results in excessive estrogen, leading to bigger prostate, inflammation, shrunken testicles, and bone weakening in males. In females, it causes thicker uterus lining, uterine enlargement, and pituitary gland growth [36,63].

The effects of ZEN on human health have also been looked into, showing resemblances to effects seen in animals. A situation of early puberty in Puerto Rican kids aged 6 months to 8 years has been linked to ZEN exposure. Studies have also found connections between ZEN exposure and specific cancers and uterus enlargements in women [11,64]. These findings underscore the potential reproductive toxicity of ZEN and its relevance to human health.

### 3.1.9 Effects on reproduction in farm animals

Studies involving rodents and livestock have discovered that ZEN can inflict damage upon their reproductive systems. To elaborate, their reproductive organs go through alterations, resulting in

a decline in fertility, reduced litter sizes, and lighter offspring [26]. ZEN has also been associated with specific adverse consequences in particular animals. In the case of male pigs, it can induce feminization, while among cows, it is tied to issues such as infertility, heightened estrogen levels, and diminished milk production [64]. Intriguingly, pigs and sheep appear to display greater susceptibility to the detrimental effects of ZEN compared to rodents [65]. The primary factor underpinning the reproductive challenges caused by ZEN lies in its capacity to imitate estrogen, thereby disturbing the inherent hormonal equilibrium and processes linked to reproduction [66].
Of all farm animals, young piglets are the most sensitive to ZEN regarding effects on the reproduction [13]. This can be explained by the fact that in pigs ZEN to a large amount is metabolized to α-ZEL [47]. This metabolite possesses a higher binding affinity to the estrogen receptors compared to ZEN, resulting in an increased estrogenic response [67]. As early as 1928, an occurrence of hyperestrogenism characterized by swelling and erythema of the vulva and uterine prolapse in gilts and atrophy of testicles and enlargement of nipples in boars could be associated with the consumption of ZEN contaminated cereals [68]. ZEN has also shown to exert estrogenic effects in other farm animals such as ruminants, sheep and poultry [6,36,69]. Even marine animals exhibit hormonal changes after ZEN exposure [70].

*3.1.9.1 Pigs*

The term hyperestrogenism describes, among other things, clinical signs of prolonged estrus, atrophy of ovaries and testicles, swollen external genitals, infertility, enlargement of the uterus and altered lactation [71].
The presence of ZEN concentrations between 0.06 - 1 mg/kg in feed over a long period causes a significant change in the reproductive tract of immature piglets, characterized by swelling and reddening of the vulva [50,55,72]. This effect is more pronounced as the concentration of ZEN increases and shows a medium to high degree swelling of the vulva in almost all animals even at ZEN concentrations of 0.42 mg/kg feed [55]. With increasing amounts of ZEN added to the feed, this effect is more prominent and is visible after only a few days [20,73]. Reports imply that after the addition of ZEN to feed is terminated, redness and swelling subside within 7 days and decrease to the level of the control group [19,20]. Apart from the appearance of edema and redness, permanent changes in the vulva may also occur. Numerous studies have shown that an increased growth of vulva length, vulva width, vulva height and vulva area could be observed by the administration of 1.1 mg/kg ZEN [74,75]. Even a single administration of 5 mg/kg ZEN resulted in a fourfold increase in the reproductive tract (uterine horns, cervix, vagina) compared to the control group [50].

A further indication for the occurrence of hyperestrogenism is an enlargement of the uterus. A significant increase in uterine weight in piglets and pigs can be observed by the intake of ZEN-contaminated feed [21,75]. The lowest ZEN concentration required to cause this effect was 17 µg/kg bodyweight for 35 days. This demonstrates that long-term exposure of low concentrations might have the same effect as a short-time exposure with higher concentrations [76]. At higher ZEN levels of 0.42 mg/kg food, a 50 % uterine enlargements could be observed [55,77]. This described hypertrophy of the uterus is associated with cellular proliferation and edema in the uterus [78]. Besides, after the addition of 6 and 9 mg/kg ZEN, a doubling of the myometrium and endometrium of the uterus was observed, caused by hypertrophy and hyperplasia [73].

In pregnant gilts, an opposite effect occurs, specifically a decrease in uterine weight. Impaired development of the uterus during pregnancy may subsequently lead to restricted growth of the placenta and ultimately to disturbed fetal growth [75]. Impaired foetogenesis could be observed by adding 60 - 90 mg of ZEN per kg of feed [72]. This could be explained by hormonal imbalance. The influence of ZEN on the hormonal pattern is dose dependent. With small amounts of ZEN, an increased progesterone concentration in the serum occurs. However, administration of ZEN at concentrations higher than 30 mg/kg of feed results in a significant decrease in progesterone concentration [72]. The ZEN metabolites α- and β-ZEL were able to inhibit follicle-stimulating hormone (FSH)- progesterone synthesis at concentrations of 15 µM in porcine granulosa cells [79]. Disturbed progesterone balance during pregnancy may lead to incomplete growth of the placenta [80].

Several observations have shown that ZEN leads to a decrease in serum concentrations of luteinizing hormone (LH) and FSH [55,81,82]. This could subsequently lead to impaired follicular development, potentially leading to a decrease in ovarian weight [55]. Such a reduction in ovarian weight has been observed in young gilts by the addition of 20 mg/kg ZEN to feed [83].

The data on the influence of ZEN on the onset of puberty, which is initiated with the first estrus, seems somewhat contradictory. Rainey *et al.* could observe that the onset of puberty in feed contaminated with ZEN was significantly earlier compared to the control group [20]. Whereas Edwards *et. al* described that the onset of puberty was delayed by ZEN [18]. The difference between these two studies depends on age and the amount of ZEN supplied. The addition of 2 mg/kg ZEN to 70-day-old piglets resulted in a significantly earlier onset of the first estrus and larger amounts of ZEN in 140-day-old pigs resulted in a delayed onset of puberty [18,20].

Data after piglets have already received their first estrus are more conclusive. In sexually mature pigs, exposure of ZEN shows a marked effect on estrus. Starting at concentrations of 3.6 mg/kg ZEN, a significant influence on the occurrence of cycle behavior can be observed. In 45 % of the gilts the return of estrus was delayed. With increasing ZEN concentrations the inter-estrus intervals are delayed even more drastically and affect a higher percentage of animals [18]. Another study showed that the addition of 4.3 mg/kg of ZEN to the feed after puberty caused 45 % of the gilts to develop a pseudo-pregnancy and prevent them from ovulating. This was additionally confirmed by the fact that no corpora albicantia was found [75]. Pseudopregnancies were demonstrated by further studies, where only 3 mg/kg ZEN were necessary to induce a delay in estrus in 25 % of the sexually mature gilts and at concentrations of 6 mg/kg only 15 % of the piglets, even though postponed, achieved a second estrus. A further indicator for the occurrence of pseudopregnancies was confirmed by increased progesterone concentrations, which under normal circumstances are only increased in pregnancies for a longer period [21]. After discontinuing a ZEN diet, it can take up to 9 weeks for plasma progesterone concentrations to drop to basal levels and to return to a regular estrus cycle [21]. These results suggest that a ZEN-restricted diet leads to a regeneration of the reproductive tract.

The effects of ZEN on the fertility of gilts show a clear trend. The addition of ZEN resulted in a significant decrease in fertility. In the further framework of this study, it was observed that the fetal weight was reduced by 24 % and the weight of the placenta by 44 % in pregnant gilts [75]. Both the fetus and placenta are responsible for the nutritional supply of growing embryos [84] and could be an explanation for the reduced litter size caused by ZEN. The consumption of ZEN is also associated with a reduced fetus which in turn can lead to stillbirths [85]. However, such an incident is not proven yet by randomized controlled trials.

*3.1.9.2 Ruminants*

Cattle show the greatest resistance to ZEN exposure of all livestock. Negative effects on the reproductive system occur only in rare cases and only with ZEN exposure over a longer time period or with very high doses of ZEN. In cows, increased ZEN concentrations cause symptoms such as hyperestrogenism, infertility and reduced milk production. For example, the addition of 14 mg/kg ZEN was able to cause infertility in cattle and dairy cows. Addition of higher amounts such as 25 and 100 mg/kg ZEN for a period of 43 days caused swelling of the external genitalia, but signs of estrus cycle alteration did not occur even at such high concentrations. In immature dairy cows, enlarged mammary glands were observed after ZEN exposure. Furthermore, a

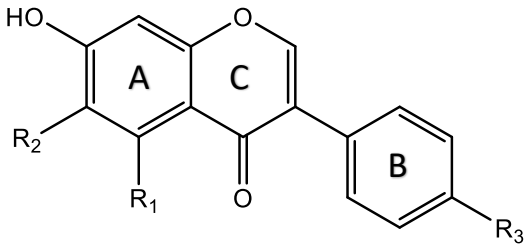
significant decrease in conception rate was observed in dairy cows administered ZEN at concentrations of 250 mg/day over a period of three estrus cycles [86].

Elevated levels of ZEN, as determined by urinary concentrations in female cattle, are associated with reduced levels of anti-mullerian hormone (AMH) [87]. AMH has, among other properties, the ability to inhibit follicle growth stimulated by follicle-stimulating hormone and is produced exclusively by the granulosa cells of the ovarian follicles of adult females [88,89].

Studies suggest that ZEN concentrations below 1 mg/kg do not affect embryo production and ovarian response after induced superovulation [90]. However, it should be noted that the concentrations under 1 mg/kg may be too low to induce a negative effect on the reproductive system.

## 3.2. Isoflavones

Phytoestrogens are natural substances found in plants, like ISF, lignans, coumestans, and stilbenes. Their polyphenolic compound structure resembles E2. Researchers have looked into these compounds for their possible ability to support chemoprevention, particularly in the development of breast cancer. However, there is some debate because they may act as agonists or antagonist regarding receptor activation, which could potentially be an additional health risk [91].




| Isoflavone | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Daidzein (DAI) | H | H | OH |
| Genistein (GEN) | OH | H | OH |
| Glycitein (GLY) | H | $OCH_3$ | OH |

Figure 5: General structure of isoflavones (ISF)

ISF are mainly present, in soybeans and red clover, containing amounts of ISF that are physiologically relevant after digestion. Asian and Central American communities have been consuming these products in higher amounts during the last decades. However, in times with the increased adoption of vegetarian and vegan diets soy protein has emerged as a favored substitute for meat in Western countries, thus potentially modifying typical exposure pattern. Furthermore, soybeans are also preferred as a protein source in animal feed [92].

### 3.2.1 Structure and biochemistry

ISF possess a basic backbone of 3-phenyl-1,4-benzopyrone, comprised of a benzene ring (A) adjacent to a heterocyclic one (C), forming a benzopyran derivative with a substituted ketone on the pyran ring and another benzene ring (B) attached to C3 [93].

ISF might appear in its free form or conjugated: aglycones (like GEN, daidzein (DAI), and glycitein (GLY)), β-glucosides (genistin, daidzin, and glycitin), acetylglucosides (acetylgenistin, acetyldaidzin, and acetylglycitin), and malonylglucosides (malonylgenistin, malonyldaidzin, and malonylglycitin) [94].

When it comes to the aglycones, GEN (4',5,7-trihydroxyisoflavone) and DAI (4',7-dihydroxyisoflavone) are most relevant, with GEN's additional hydroxy group at C5 contributing to increased antioxidant activity compared to DAI [95]. GLY (4',7-dihydroxy-6-methoxyisoflavone), biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), and formononetin (7-hydroxy-4′-methoxyisoflavone) are methylated derivatives, with the latter two serving as precursors of GEN and DAI [93].

Equol (EQ), a gut bacterial metabolite of DAI, is considered a phytoestrogenic ISF. Its discovery in the 1930s gained significance when linked to the "clover disease" affecting sheep grazing on subterranean clover pastures in Western Australia, leading to reproductive issues, including infertility [16].

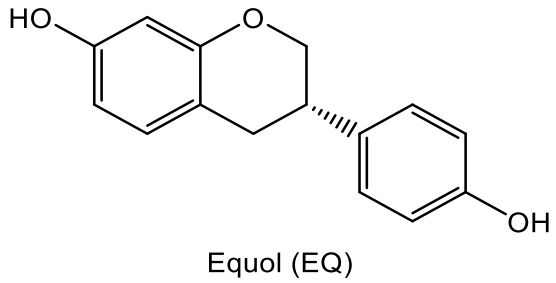



Figure 6: Chemical structure of equol (EQ)

Unlike aglycones EQ (7 hydroxy 3 (4' hydroxyphenyl) chroman) stands out due, to its heterocyclic ring that contains a chiral center at C3. Interestingly despite lacking elements EQ showcases higher antioxidant activity compared to DAI. The exact reasons for this disparity are still not fully understood [95]. It is likely that the distinct conformations of its isomers (R (+) EQ and S (-) EQ) play a role in influencing their interactions, with the ligand binding domain of estrogen receptors [25].

### 3.2.2 Occurrence

ISF are abundant in plant tissues of the Leguminosae (Fabaceae) family and Papilionidae subfamily, especially in germ/seeds and sprouts, serving as defense mechanisms against microbial plant pathogens [96].

Figure 7: Picture of soybeans, credit: United soybean board (https://commons.wikimedia.org/wiki/Category:Soybean_cultivation#/media/File:Close-up_of_High_Oleic_Soybean_Pods_(10872288796).jpg )

In soybeans, vacuoles store malonyl derivatives of genistin, daidzin, and glycitin, while non-fermented soy foods contain β-glucoside conjugates. Enzymes like glucosyl- and malonyltransferases mediate the conversion from ISF to their conjugates, and fermentation leads to sugar moiety loss [97]. The content and composition of ISF in food products can vary due to factors like genotype, soil, altitude, temperature, postharvest processing, and different techniques [98]. ISF content in soybeans ranges between 1.3 and 9.5 g/kg, with the order GEN>DAI>GLY in aglycone content [99]. In Austrian dairy pastures, ISF range from 8 to 129 µg/kg dry weight, influenced by environmental temperature [100]. Industrial treatments reduce β-glucoside ISF by up to 37 %, while aglycone content remains stable [101]. Red clover is rich in ISF, particularly formononetin and biochanin A, followed by GEN and DAI [102]. Red clover sprouts contain higher ISF levels compared to other clover species, ranging from 2 to 4 g/kg [103].

### 3.2.3 Microbial transformation

In the human gastrointestinal tract (GIT), glycosylated ISF face obstacles in passive diffusion due to their size and hydrophilic structure. Their bioavailability depends on specific transporters or enzymatic hydrolysis of sugar moieties. Gut microflora's β-glucosidases and enterocyte's lactase phlorizin hydrolase are involved in deglycosylation of ISF glycosides to aglycones like GEN and DAI, which are then absorbed faster in the gut epithelium and further metabolized in the bloodstream [104].

Aglycones can also be formed through O-demethylation of methoxyl derivatives by *Eubacterium limosum* in the human intestinal tract. In the human gut microbiota, bacterial strains facilitate reductive metabolization of GEN and DAI, leading to the production of metabolites like dihydro-GEN (DHG), 6'-hydroxy-O-demethyl-angolensin (6-OH-ODMA), tetrahydro-GEN (THG), and 5-hydroxy-EQ (5-OH-EQ) [92].

DAI follows a similar metabolic pathway, being reduced to dihydro-DAI (DHD) and further transformed to O-demethyl-angolensin (ODMA) or tetrahydro-DAI (THD), with THD enhancing estrogenicity. EQ isomers are bioavailable, with human intestinal bacterial synthesis mainly producing S-EQ. The ability to produce EQ varies between species, with higher percentages in Asian countries due to greater soy consumption. The explanation for this variability remains unclear and may involve dietary and non-dietary factors, affecting the human microbiome [92].

In contrast, almost all animal species studied can produce EQ, with variations in plasma concentrations observed among different species. For instance, cattle have higher EQ levels compared to sheep [92,105].

### 3.2.4 Metabolism

ISF aglycones undergo phase I metabolism, involving cytochrome P450-dependent monooxygenases (CYP), in the intestine or liver after crossing the gastrointestinal barrier. In the hepatic metabolism of GEN and DAI, CYP1A2 plays a significant role, with minor contributions from CYP2E1, CYP1A1, and CYP1B1 [106].

Rat liver microsome studies revealed that GEN and DAI produced multiple hydroxylated products, with six and nine metabolites, respectively. Monohydroxylated forms further transformed into di- and tri-hydroxylated compounds. EQ metabolism in rats showed the production of eleven metabolites, including mono- and dihydroxylated compounds, with 3'-hydroxy-EQ being the main metabolite [107].

Human liver microsome studies identified three monohydroxylated and three dihydroxylated metabolites of DAI, with significant amounts of five metabolites detected in urine during *in vivo* experiments. For GEN, six hydroxylated products were formed, and five were detected in human urine samples [108]. Human liver microsomes produced six EQ metabolites, with 3'- and 6-hydroxy-EQ as dominant ones, and the 4-hydroxy-EQ was also detected in human urine samples [107].

Phase II metabolic pathways of ISF involve enzymatic conjugation reactions in the small intestine and liver. Uridine-5'-disphosphate glucuronyltransferases (UGTs) mediate glucuronidation by adding glucuronic acid to ISF, forming glucuronides. Sulfotransferases (SULTs) catalyze sulfation by transferring sulfate groups to ISF. These reactions increase ISF' polarity, aiding in their excretion and detoxification [109]. In humans, seven common phase II metabolites are identified for GEN and DAI, and eight for EQ, formed mainly at the C7 and C4' positions [110].

Studies on human and rodent blood samples have shown that the main metabolites for GEN in humans are GEN-7-glucuronide-4'-sulfate (GEN7G4'S) and GEN-4'-7-diglucuronide (GEN4',7dG). Rodent species exhibit differences in metabolite concentrations, with gender-specific variations in rats and multiple major metabolites in mice [111]. For DAI, major metabolites in humans are similar to GEN, but differ in rodents, with GEN-4'7-disulfate (GEN4',7dS) being the main metabolite in rats and only single conjugates in mice [111]. In human plasma samples, the presence of EQ metabolites depends on individual capabilities for EQ production. Rats mainly produce EQ -7-glucuronide (EQ7G), regardless of gender, with EQ-4'-sulfate (EQ4'S) found only in male rats. Mice produce both EQ7G and EQ4'S [111]. Farm animals, such as sheep and cattle, display differences in conjugative activity in the gastrointestinal tract, with glucuronidation being the primary conjugation reaction for DAI and EQ [92,105].

### 3.2.5 Enterohepatic circulation and excretion

In rats, enterohepatic circulation has been noticed for GEN and its metabolite GEN-7-glucuronide (GEN7G), where substances are absorbed from the intestine, pass through the liver, and are excreted into bile, which can be reabsorbed into the small intestine and transported back to the liver [112]. However, research on this topic is limited, and most studies focus on urinary excretion. In human urine samples, around 70 % of GEN and DAI glucuronides were detected, followed by 20 % of sulfate conjugates and only traces of the aglycones [113]. In cows, the conjugated forms of ISF were excreted in urine, while the aglycones were excreted in feces. Milk samples of lactating ruminants also contain varying concentrations of ISF, especially EQ [114]. In pigs, glucuronides of GEN and DAI were mainly excreted in urine. Rats were capable of excreting 40-

50 % of the aglycones. EQ was also found in rat urine samples, primarily as glucuronides and in lower concentrations as the aglycone [115].

### 3.2.6 Estrogenicity and health effects

ISF share structural similarities with E2, allowing them to interact with ERs and modify ER structure, as well as interact with coactivators. Depending on tissue specificity and the ratio of ERα/ERβ, ISF may act as partial agonists or antagonists, making them potential natural selective estrogen receptor modulators (SERMs) [116]. ISF have a higher affinity for ERβ, which is not the primary receptor in certain tissues like the uterus, enabling targeted actions in specific tissues while avoiding certain estrogenic effects [117].

GEN exhibits a 40-fold selectivity for ERβ and adopts an antagonistic conformation despite comparable binding to E2, suggesting the significance of the transcriptional response to the ligand [118]. EQ prefers ERβ binding, specifically for the S-enantiomer, while R-EQ prefers ERα. Both their affinities are higher than their precursor DAI, and the metabolic transformation of DAI to ODMA reduces estrogenicity considerably [119].

Phase II metabolism was initially thought to detoxify parent compounds, making ISF conjugates non-estrogenic. However, DAI and GEN 7-*O*-glucuronides have been found to exert weak estrogenic activity in mouse uterine cytosolic ERs, with G7G showing higher activity than D7G [120]. Conversely, sulfation at the 7-position for DAI significantly increases estrogenic activity, while sulfation in the same position for EQ and GEN reduces estrogenic activity in human breast cancer cells [121]. Limited information is available regarding the majority of ISF metabolites and their estrogenic effects. Further research is required to understand the diverse effects of ISF and their metabolites on estrogenicity and health outcomes.

### 3.2.7 Humans

Phytoestrogens have been linked to potential cancer risk in estrogen-sensitive tissues based on *in vitro*, animal, and epidemiological studies. However, clinical trials have mainly focused on the positive effects of phytoestrogens on bone metabolism, cardiovascular diseases, obesity, brain function, and various types of cancer [91].

One randomized controlled trial investigating the effects of soy protein with phytoestrogens on cognitive function, bone mineral density (BMD), and plasma lipids in postmenopausal women over one year did not show significant improvements [122]. Similarly, another one-year trial in early postmenopausal women did not find positive effects of consuming phytoestrogen-rich products on BMD, bone metabolism, and hormonal status [123]. Studies reporting favorable

results often involve Asian populations with a long-term soy diet, particularly during early childhood, and the ability to metabolize DAI to EQ [124].

Long-term studies in rodents, using rodent tumor models to assess the impact of phytoestrogens on breast cancer, have shown mostly non-adverse effects compared to control animals, with some demonstrating preventive effects when phytoestrogen exposure occurred during prepubescence. However, conflicting results were observed in studies on the effects of phytoestrogens on pre-existing tumors and cancer cells, with GEN appearing to stimulate the growth of pre-existing estrogen receptor-positive breast cancer cells [125].

However, comparing these animal studies to human studies requires considering differences in physiology, particularly in metabolism. Rodents and humans differ in their ability to produce EQ from DAI and in the absorption and distribution of different metabolites. This highlights the importance of further investigating these substances to better understand their effects on different types of cancer cells.

### 3.2.8 Pigs

Feeding red clover silage were able to induce signs of hyperestrogenism such as fertility problems in pigs. The underlying effects were caused by the high content of ISF, substances that have estrogenic properties [126]. Further feeding trials of 10-week-old piglets with soy showed that the soy-containing diet was able to induce estrogenic effects in the piglets. Again, symptoms of hyperestrogenism such as swelling of the mammary gland and vulva, enlargement of the uterus and pathological changes of ovaries were observed [127].

In pregnant sows, the influence of DAI on neonatal piglets was investigated. Compared to the control group, the male newborn piglets whose mothers were fed with DAI had a significantly higher birth weight. No other influences on the piglets were observed. However, it has been observed that the addition of GEN and DAI to the feed of lactating sows caused estrogenic effects in the piglets. Therefore, it can be concluded that ISF through breast milk lead to an exposure of the estrogenic substances to the piglets. This exposure caused swelling and reddening of the vulva in female piglets [128].

### 3.2.9 Ruminants

A series of pathological-anatomical examinations in animals fed mainly with red clover showed signs of hyperplasia and cervicitis in the endometrium of sheep. Chronic infertility was observed in sheep after continuous exposure to ISF, reflected by symptoms that included irreversible changes in estrogen-sensitive organs and completely masculinized sheep [129].

In cattle, only temporary infertility has been observed so far due to ISF exposure. This is reflected by a reduced conception and ovulation rate [129]. In addition, studies have reported other symptoms such as irregular estrus cycles, vulvar swelling, cyst formation after egg death, and behavioral abnormalities such as anestrousness and nymphomania. After discontinuation of ISF exposure, symptoms decrease over a few days to weeks and disappear with progressive time [129]. Cattle are less sensitive to exposure to ISF compared to other ruminants and this is suspected to be due to differences in estrogen receptors and tremendous weight differences [105]. Formononetin and DAI, which are converted in the rumen into the highly estrogenic EQ, are mainly responsible for the increasing infertility [130]. However, the ISF GEN and biochanin have also been implicated in infertility [131]. Since soy is one of the most widely used protein sources, an adverse effect on the reproductive system due to the high content of ISF cannot be excluded. In fact, an influence of soy-rich diets on female reproductivity in cows can be observed. Among soy-fed cows, only three out of five cows became pregnant after successful insemination, that is correlated to EQ where elevated concentrations has been found in the blood [132].

## 3.3 Zearalenone and isoflavones co-occurrence

To evaluate potential synergistic effects between the mycoestrogen ZEN and ISF, their co-occurrence in various environmental, dietary, and animal feed sources needs investigation. A study in the Douro River estuary in Portugal found DAI and GEN in seasonal water samples, with concentrations up to 277.4 ng/L and 130.0 ng/L, respectively, during summer and spring. ZEN concentrations remained below 137.5 ng/L during the same periods, with likely sources being agricultural effluents due to nearby farming activities [133].

Similarly, an Austrian survey of pasture field samples in 2019 showed approximately half of the samples contaminated with ZEN, averaging 29.6 µg/kg. The samples also contained GEN, DAI, and GLY with average concentrations of 2760, 936, and 7470 µg/kg, respectively. ZEN levels remained below the EU guidance value of 500 µg/kg for dairy cattle and sheep feed [100].

Further research in 2021 revealed the co-occurrence of ZEN with ISF in various livestock feed products, being most prevalent in poultry feed (≈ 55 %), followed by pig feed (≈ 30 %), and cattle feed (≈ 15 %). Some samples exceeded the ZEN concentration guidelines set by the European Commission, particularly in pig feed [92].

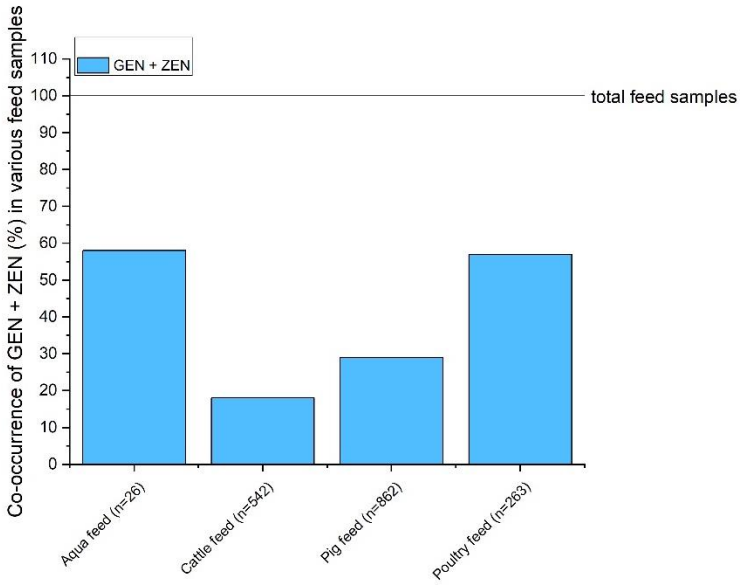



Figure 8: Co-occurrence of genistein (GEN) and zearalenone (ZEN) in various feed

A study on soy-based burgers found DAI to have the highest average concentration at 1250 µg/kg, followed by GLY at 570 µg/kg, and GEN at 170 µg/kg. ZEN, however, was not detected in any of the analyzed samples [134]. While data on the co-occurrence of ZEN and ISF in the human diet is limited, studies on animal feed products indicate the potential simultaneous presence of both estrogenic compounds.

## 3.4 Mode of action

ISF and ZEN, along with their metabolites, have the ability to mimic the actions of E2 due to their structural similarities with this hormone. Figure 10 illustrates this structural resemblance, particularly emphasizing the phenol ring.

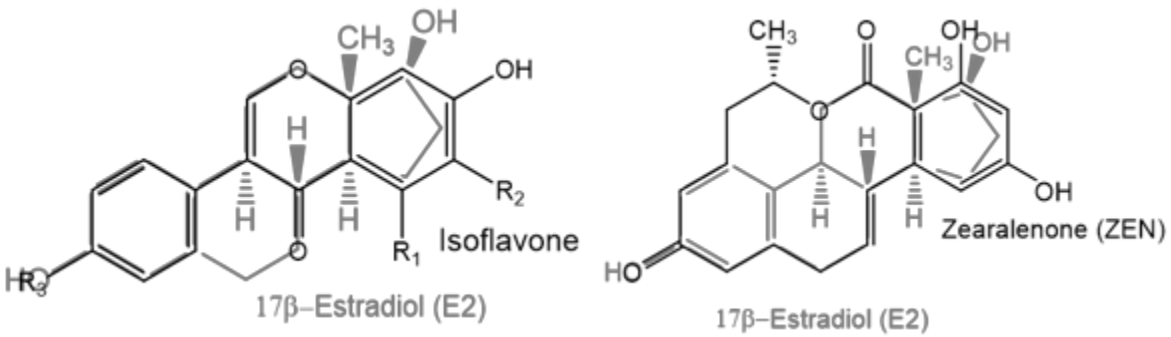



Figure 9: Overlay of ISF or ZEN with 17-β-estradiol (E2) to show the structural similarity.

### 3.4.1 Estrogen receptors

E2 is a crucial steroidal hormone that regulates cell growth, differentiation, and various physiological functions by binding to ERs, which are intracellular receptor proteins belonging to the nuclear hormone receptor (NHR) family. ERs function as ligand-activated transcription factors and consist of six distinct domains: A/B (amino terminal domain), C (DNA-binding domain or DBD), D (flexible hinge with nuclear localization signals), E (ligand-binding domain or LBD), and F (carboxyl-terminus) (see Figure 6).

ERα has 595 amino acids, while ERβ has 530 amino acids, and both receptors share high homology in their DNA-binding domains (DBD). The A/B domain is the amino terminal region, C is the highly homologous DBD, and D serves as a flexible hinge with NLS, connecting the C domain to the E domain. The E domain functions as the ligand-binding domain, facilitating hormone binding and interactions with ligand-dependent co-regulatory functions like the activation function AF-2. The role of the F domain in ERβ is not fully understood, but in ERα, it appears to modulate transcriptional activity and enhance receptor stability [135].

ERα is predominantly found in the uterus, mammary glands, pituitary gland, skeletal muscle, bones, and adipose tissue, while ERβ is located in the ovary, prostate, lung, cardiovascular system, and central nervous system. Both receptors are co-expressed in the thyroid, uterus, and brain, where ERβ often counteracts the effects of ERα [136].

### 3.4.2 Binding to ER: ERE-dependent pathway

Ligand binding to estrogen receptors can activate various signaling cascades through two main pathways: the estrogen response element (ERE) dependent pathway and the estrogen response element independent pathway. The ERE is a DNA region with a consensus sequence, but most estrogen-sensitive genes have variations of this sequence [136].

Estrogen receptors can be found in various cellular locations, including the nucleus, cytoplasm, plasma membrane, and mitochondria. When E2 or another ligand binds to ER, the ER dissociates from heat-shock protein (Hsp) complexes. In the ERE-dependent pathway, ligand binding induces dimerization in the ligand binding site of ER, allowing it to attach to specific EREs on the DNA through the DNA-binding domain of the receptor.

Ligand binding also leads to conformational changes in the ER complex, exposing new binding surfaces. Coactivators can then bind to these surfaces and participate in the process of chromatin

untangling. Coactivators like histone acetylases (HATs) facilitate the acetylation of histones, making certain areas of chromatin more accessible for transcription initiation.

On the other hand, corepressors can suppress transcriptional activity by recruiting histone deacetylases (HDACs), which act repressively on chromatin. ER antagonists demonstrate such effects, resulting in reduced translation of specific proteins [137].

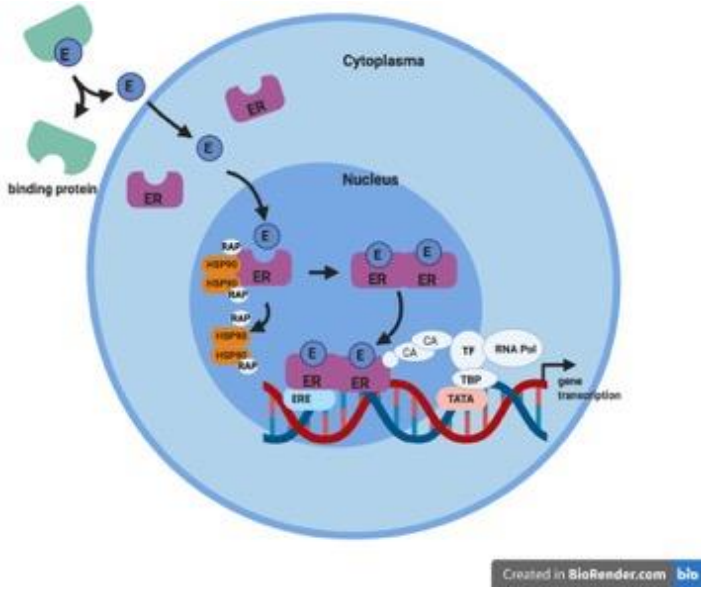



Figure 6: Illustration of the cellular estrogen receptor signaling pathway. Created with BioRender.com. See text for a description of the cascade. E= estrogen, ER= estrogen receptor, HSP90 = heat shock protein, RAP= receptor associated protein, ERE= estrogen response element, TF= transcription factors, TBP= TATA binding protein. [137]

### 3.4.3 Binding to ER: ERE-independent pathway

Approximately 30 % of genes regulated by estrogen receptors ERs lack the ERE consensus sequence, suggesting an alternative regulatory mechanism [138]. One alternative pathway is mediated through protein-protein interactions, also known as "transcriptional crosstalk," where the transcribed gene does not require an ERE sequence. These includes protein-protein interactions involving estrogen receptors. E2 interacts with Jun and Fos proteins at the activation protein 1 (AP-1) binding site. The activation function (AF) domain on the ER stabilizes the protein complex, enabling the recruitment of general transcription coactivators at the promoter region for subsequent transcription of specific estrogen-responsive genes. Furthermore, the transcription factor Sp1 is able to interact with ERs and activates GC-rich motifs located in the promoter regions of estrogen-responsive genes [139]. However, these mechanisms can also act in a reversible manner, exerting repressive effects on transcription. Recent research has revealed non-genomic

mechanisms of estrogen action, occurring too rapidly to be attributed to mRNA transcription. One such mechanism involves the G-protein coupled estrogen receptor (GPER or GPER30), found intracellularly and acting alongside the classical ER [140].

One rapid non-genomic action of estrogen involves the influx of calcium ions from extracellular regions into the cell interior, activating various physiological processes [141]. This mobilization of calcium ions can activate signaling pathways like ERK1/2, MAPK, and PI3K-Akt, regulating the cell cycle, apoptosis, proliferation, growth, and differentiation [142]. Additionally, these pathways may prompt genomic actions by activating transcription factors, such as AP-1 [140].

### 3.4.4 Isoflavones and Zearalenone as ER agonists

Exogenous compounds like ZEN and ISF interact with estrogen receptors, with ISF having a stronger affinity for ERβ and ZEN preferentially binding to ERα [23–25]. These compounds induce various mechanisms upon binding to estrogen receptors.

ZEN exhibits estrogenic effects, including gene induction, cell proliferation, and activation of the ERK 1/2 pathway, similar to E2, even at low concentrations [143]. It also upregulates certain steroid hormone receptors, resembling classical estrogenic responses [67]. Furthermore, ZEN activates ERE-mediated transcription [144].

ISF, particularly GEN, have a strong binding affinity for ERβ and promote estrogen-dependent cell proliferation. They can also interfere with E2-induced mechanisms when combined with E2, suggesting interaction between ER isoforms [145]. GEN selectively triggers the transcriptional pathways of ERβ [146]. Activation of estrogen receptors by these exogenous estrogens leads to a wide range of outcomes, highlighting the potential interplay between ER isoforms.

# 4. Overview of publications and manuscripts and key results

## 4.1 Publication 1 (review article)

| | |
|---|---|
| status: | published |
| title: | Isoflavones in Animals: Metabolism and Effects in Livestock and Occurrence in Feed |
| authors: | Dino Grgic [1], Elisabeth Varga [1], Barbara Novak [2], Anneliese Müller [2] and Doris Marko [1] |
| affiliations of authors: | **1:** University of Vienna, Department of Food Chemistry and Toxicology, Waehringer Str. 38, A-1090 Vienna, Austria<br>**2:** BIOMIN Research Center, Technopark 1, 3430 Tulln, Austria |
| published in: | Toxins, Volume 13, Issue 12 |
| year: | 2021 |
| accepted for publication: | November 19th, 2021 |
| DOI | https://doi.org/10.3390/toxins13120836 |
| author´s contribution: | Conceptualization: D.G., E.V., B.N. and D.M.; writing – original draft preparation: D.G.; writing – review editing: E.V., B.N., A.M. and D.M.; visualization: D.G. Funding acquisition: EV, BN and DM; Resources: BN, DM; Supervision: EV, BN and DM. |

## 4.2 Publication 2

| | |
|---|---|
| status: | published |
| title: | Estrogenic in vitro evaluation of zearalenone and its phase I and II metabolites in combination with soy isoflavones |
| authors: | Dino Grgic [1,2], Andrea Betschler [1], Rebeka Früholz [1], Barbara Novak [3],Elisabeth Varga [1] and Doris Marko [1] |
| affiliations of authors: | **1:** University of Vienna, Department of Food Chemistry and Toxicology, Waehringer Str. 38, A-1090 Vienna, Austria<br>**2:** Doctoral School in Chemistry, University of Vienna, Währinger Str. 38-40, 1090 Vienna, Austria<br>**3:** BIOMIN Research Center, Technopark 1, 3430 Tulln, Austria |
| published in: | Archives of Toxicology, Volume 96, Page 3385 - 3402 |
| year: | 2022 |
| accepted for publication: | August 03rd, 2022 |
| DOI | https://doi.org/10.1007/s00204-022-03358-3 |
| author´s contribution: | Conceptualization: DG, EV, BN and DM; Methodology: DG, EV and DM; Formal analysis and investigation: DG, AB and RF; Writing—original draft preparation: DG; Writing—review and editing: AB, RF, EV, BN and DM; Funding acquisition: EV, BN and DM; Resources: BN, DM; Supervision: EV, BN and DM. |

Key results:

In this study, the combined effects of certain compounds found in soy (like: genistein (GEN), glycitein (GLY), daidzein (DAI) and its gut microbial metabolite equol (EQ)) and corn (zearalenone (ZEN) and its metabolites) that exhibit estrogenic properties were investigated. Previous research has shown that these compounds, which include both natural plant compounds (phytoestrogens) and fungal toxins (mycoestrogens), can potentially interact and amplify their estrogenic effects. The goal was to understand how these compounds, commonly present in animal feed, might influence estrogenic responses.

In this study, when mycoestrogens were combined with certain phytoestrogens (GEN, DAI, EQ) at low concentrations, the estrogenic effects were significantly enhanced. However, at higher concentrations of phytoestrogens, the interactions became less significant, suggesting a complex relationship between these compounds.

Furthermore, the results indicated that the way these compounds interacted with estrogen receptors in cells influenced their effects. For instance, ZEN and its metabolites are able to interact with both types of estrogen receptors (ERα and ERβ), while phytoestrogens preferably bind to ERβ. This selectivity of these substances in binding to different estrogen receptors might be a crucial factor for synergistic estrogenic effects.

This study emphasizes the importance of considering not only the individual effects of these compounds but also their interactions, especially in scenarios where these substances are consumed together, as in the case of animal feed. This study underscores the need for a more comprehensive understanding of how these compounds interact in the body, which is vital for ensuring the safety of consumers and animals exposed to these substances.

## 4.3 Publication 3

| | |
|---|---|
| status: | published |
| title: | Estrogen receptor α interaction of zearalenone and its phase I metabolite α-zearalenol in combination with soy isoflavones in hERα-HeLa-9903 cells |
| authors: | Dino Grgic [1,2], Barbara Novak [3],Elisabeth Varga [1,4] and Doris Marko [1] |
| affiliations of authors: | **1:** University of Vienna, Department of Food Chemistry and Toxicology, Waehringer Str. 38, A-1090 Vienna, Austria<br>**2:** Doctoral School in Chemistry, University of Vienna, Währinger Str. 38-40, 1090 Vienna, Austria<br>**3:** dsm-frmenich, ANH R&D center, Technopark 1, 3430 Tulln, Austria<br>**4:** Unit Food Hygiene and Technology, Institute of Food Safety, Food Technology and Veterinary Public Health, University of Veterinary Medicine, Vienna, Veterinärplatz 1, 1210 Vienna, Austria |
| published in: | Mycotoxin Research, 2023 |
| year: | 2023 |
| accepted for publication: | October 05th, 2023 |
| DOI | https://doi.org/10.1007/s12550-023-00506-1 |
| author´s contribution: | Conceptualization: DG, EV, BN and DM; Methodology: DG, EV and DM; Formal analysis and investigation: DG; Writing—original draft preparation: DG; Writing—review and editing: EV, BN and DM; Funding acquisition: EV, BN and DM; Resources: BN, DM; Supervision: EV, BN and DM. |

Key results:

In this study, the combined effects of mycoestrogens (specifically zearalenone (ZEN) and α-zearalenol (α-ZEL)) and isoflavones (ISF) on the estrogen receptors α (ERα) were investigated. The previous study had shown that these compounds, when present together, create synergistic estrogenic effects. However, when testing these combinations on a cell model expressing only ERα, no enhanced estrogenic effects were observed at low ISF concentrations. This suggests that the presence of both ER isoforms (α and β) is crucial for the synergistic effects seen previously.

In experiments, α-ZEL demonstrated higher potency than ZEN in inducing a specific enzyme (luciferase) related to estrogenic activity. Notably, ISF induced luciferase activity significantly at higher concentrations than ZEN and α-ZEL. However, these high luciferase levels induced by ISF were not a direct result of estrogen receptor activation. It was discovered that ISF interacted with the luciferase enzyme, stabilizing it and protecting it from degradation. Consequently, in the presence of ISF, the luciferase enzyme remained stable and produced higher activity levels over time.

This study emphasized the importance of considering the specific isoforms of estrogen receptors in understanding how myco- and phytoestrogens interact. It also highlighted the complexity of testing these interactions and the need for caution when interpreting results, especially in assays that may not accurately reflect the biological processes happening in the body.

# 5. Summary of the key results and conclusion

In recent discussions regarding the safety of food and animal feed, it has become increasingly evident that assessing the risk of exposure to various compounds necessitates a more comprehensive approach. Rather than simply evaluating the individual effects of substances, it is imperative to consider how these compounds interact with one another and their combined impact on human and animal health. This holistic perspective is crucial in providing reliable risk assessments, particularly in cases where substances exhibit toxic or bioactive properties.

One area of concern lies in the realm of estrogenic compounds. Xenoestrogens, for instance, have been demonstrated to potentiate their toxic effects when studied *in vitro*. Moreover, the co-occurrence of mycoestrogens and phytoestrogens in animal feed commodities has been increasingly documented. Soy, a prevalent protein source in animal feed, contains three major isoflavones (GEN, DAI, and GLY), while corn, a common carbohydrate additive, often harbors the mycotoxin ZEN. Both ISF and ZEN are known for their estrogenic properties, albeit at different concentration thresholds.

To delve deeper into this complex interplay, a study was conducted to investigate the estrogenic effects of these compounds, both individually and in combination. Results demonstrated that the mycotoxin

α-ZEL exhibited the highest estrogenic potency, followed by α-ZAL and ZEN. Surprisingly, ZEN-14-S, a phase II metabolite of ZEN, did not display estrogenic potential under the study's conditions. Importantly, the study found that ISF required significantly higher concentrations (100 to 1000 times) to induce estrogenic responses compared to ZEN and its metabolites.

Among the ISF, GEN emerged as the most potent, followed by EQ, DAI, and GLY. Notably, higher concentrations of GEN, DAI, and EQ led to a minor reduction in ALP activity compared to lower concentrations, suggesting complex cellular mechanisms at play. These mechanisms could involve the inhibition of key kinases or the suppression of estrogen receptor expression, both of which have been documented in previous research.

Furthermore, phase II metabolism of ISF, primarily resulting in sulfoglucuronides and diglucuronides, might contribute to reduced estrogenic activity. Nonetheless, some studies indicate that these phase II metabolites still retain some biological activity, albeit to a lesser extent than their parent compounds.

This project also explored combinatory effects between mycoestrogens and ISF, revealing that most combinations induced higher ALP activity compared to single substances. These synergistic effects were most pronounced at lower mycoestrogen and ISF concentrations, with some shifts toward additive or antagonistic effects at higher ISF concentrations. Intriguingly, combinations of ZEN with ISF consistently demonstrated the most potent estrogenic effects, possibly due to the differential binding affinities of ZEN and ISF to estrogen receptors.

To delve deeper, the study extended its investigation to a different cell model expressing only ERα, which is one of the two estrogen receptor isoforms (the other being ERβ). Interestingly, in this cell model, combinations of ISF and mycoestrogens did not induce enhanced estrogenic effects, indicating that the presence of both ER isoforms might be necessary for synergistic effects.

However, it is worth noting that in some experiments, ISF displayed an unusual phenomenon called superinduction, wherein they exceeded the activities of the known estrogenic compound E2. This raised questions about whether this phenomenon was a true reflection of receptor-mediated interactions. It was suggested that ISF might interact with the reporter enzyme luciferase, stabilizing it and thereby increasing the signal intensity. Indeed, further experiments confirmed that ISF could increase the stability of luciferase, supporting the hypothesis that ISF might stabilize the enzyme and lead to high bioluminescence signals, as observed in combinations between ISF and ZEN.

In conclusion, these findings highlight the complex and often synergistic nature of estrogenic compounds. The presence of both estrogen receptor isoforms appears to be crucial for eliciting enhanced estrogenic effects, and the phenomenon of superinduction suggests the need for careful consideration of the mechanisms underlying these interactions. This comprehensive approach to risk assessment underscores the importance of evaluating the combined effects of compounds in real-world scenarios, rather than relying solely on assessments of individual substances. Ultimately, this research has implications for food and feed safety standards and emphasizes the need for reevaluating maximum allowable levels of certain compounds to ensure consumer safety.

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# 7. Abbreviations

| | |
|---|---|
| CYP | Cytochrome P450-dependent monooxygenases |
| DAI | Daidzein |
| DHG | Dihydro-GEN |
| DHZEN | Decarboxylated hydrolyzed zearalanone |
| DMSO | Dimethylsulfoxid |
| DON | Deoxynivalenol |
| ERE | Estrogen response element |
| EQ | Equol |
| GIT | Gastrointestinal tract |
| GEN | Genistein |
| GLY | Glycitein |
| HAT | Histone acetylases |
| HDACs | Histone deacetylases |
| HSD | Hydroxysteroid dehydrogenase |
| HSP | Heat-shock protein |
| HZEN | Hydrolyzed zearalanone |
| NHR | Nuclear hormone receptor |
| SERMs | Selective estrogen receptor modulators |
| SHBG | Sex hormone-binding globulin |
| SULTs | Sulfotransferases |
| THD | Tetrahydro-DAI |
| THG | tetrahydro-GEN |
| UGTs | Uridine-5'-disphosphate glucuronyltransferases |
| ZAN | Zearalanone |
| ZEN | Zearalenone |
| α-ZEL | α-zearalenol |
| β-ZEL | β-zearalenol |
| 6-OH-ODMA | 6'-hydroxy-O-demethyl-angolensin |

# 8. Publications

## 8.1 Publication 1:

“Isoflavones in Animals: Metabolism and Effects in Livestock and Occurrence in Feed”

toxins

MDPI

*Review*

# Isoflavones in Animals: Metabolism and Effects in Livestock and Occurrence in Feed

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**Abstract:** Soybeans are a common ingredient of animal feed. They contain isoflavones, which are known to act as phytoestrogens in animals. Isoflavones were described to have beneficial effects on farm animals. However, there are also reports of negative outcomes after the consumption of isoflavones. This review summarizes the current knowledge of metabolization of isoflavones (including the influence of the microbiome, phase I and phase II metabolism), as well as the distribution of isoflavones and their metabolites in tissues. Furthermore, published studies on effects of isoflavones in livestock species (pigs, poultry, ruminants, fish) are reviewed. Moreover, published studies on occurrence of isoflavones in feed materials and co-occurrence with zearalenone are presented and are supplemented with our own survey data.

**Keywords:** isoflavones; genistein; daidzein; glycitein; animal feed; pigs; ruminants; poultry; zearalenone; mycoestrogens; phytoestrogens

**Key Contribution:** The review provides an overview of the metabolism and effects of isoflavones in livestock. Furthermore, the co-occurrence of isoflavones and zearalenone and its metabolites is summarized and own occurrence data are presented.



**Citation:** Grgic, D.; Varga, E.; Novak, B.; Müller, A.; Marko, D. Isoflavones in Animals: Metabolism and Effects in Livestock and Occurrence in Feed. *Toxins* **2021**, *13*, 836. https://doi.org/10.3390/toxins13120836

Received: 18 October 2021
Accepted: 19 November 2021
Published: 24 November 2021



## 1. Introduction

Isoflavone(s) (ISF) are secondary plant metabolites belonging to the group of polyphenols [1]. They are characterized by the presence of a benzene ring attached to the third position of the carbon ring [2]. Due to structural and functional similarity with the estrogen-active hormone 17β-estradiol, ISF are classified as phytoestrogens [3]. ISF (Figure 1) are mainly found in plants of the legumin family and thus, high concentrations occur in soybeans (*Glycine max*) and red clover (*Trifolium pratense*). They reside as glycosides with low estrogenic activity compared to their deglycosylated form also referred to as aglycone. Upon ingestion, these compounds are metabolically hydrolyzed by the intestinal microflora to their aglycones thus potentially mediating an estrogenic stimulus [2,4].

Soy products are commonly used as animal feed and due to the increased global production and high protein content of about 38%, soybeans are the most favored protein provider for pig feed [5]. Soybean-based products contain primarily daidzein (DAI), genistein (GEN) as well as their conjugates and in small amounts glycitein (GLY). The overall ISF content of soybean is 1.2–4.2 g/kg dry weight according to older analysis, which agrees with a recent study where the average amount of ISF varies between 0.7 and 5.2 g/kg [6,7]. In the diet of livestock, besides soybeans also red clover is one of the main sources of ISF. The main ISF in red clover are formononetin and biochanin A [1] and ISF occur in concentrations as high as 10–25 g/kg of the dry weight [8] with formononetin accounting for 0.8–11 g/kg of the dry weight [9]. ISF concentrations depend on the plant part, growth stage, cultivar, growing conditions, and preservation method [10,11].

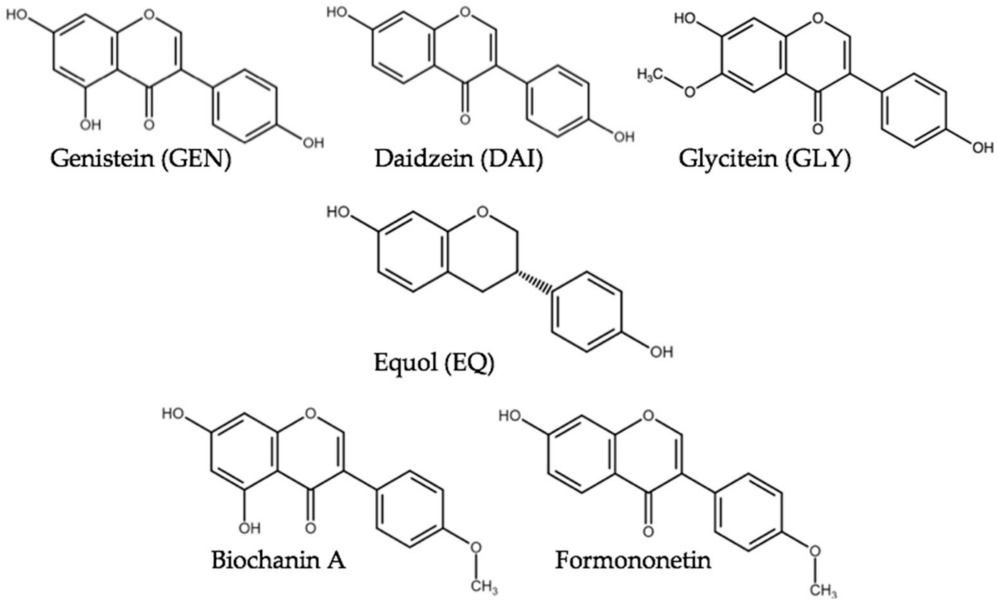


**Figure 1.** Structure of various isoflavones and equol, a microbial metabolite of daidzein.

Over the last 50 years, interest in ISF and their effects on animals has increased, especially in the field of agricultural research [12]. Studies have been conducted in various animal species including pigs, cattle, sheep, poultry, and rodents, which found positive as well as negative effects of ISF on the animal health [13–25]. Positive properties that were attributed to ISF are its growth-promoting, antioxidant and antimicrobial effects [26]. However, negative effects of ISF on the reproductive tract were observed in animals, including symptoms such as an enhanced rate of endometriosis, inability to become pregnant and abortions [27]. The first reports of negative effects of ISF on reproductive health date back to the 1940s, when reproductive problems were observed in sheep after excessive consumption of ISF-containing *Trifolium subterraneum* (subterranean clover), a condition that became known as "clover disease" [28].

In addition to phytoestrogens, other feed contaminants such as mycotoxins possess estrogenic potential. Estrogenic mycotoxins are termed "mycoestrogens". The most prominent examples of mycoestrogens are zearalenone (ZEN) and its derivatives (e.g., α-zearalenol, β-zearalenol, zearalanone, α-zearalanol, and β-zearalanol) (Figure 2). ZEN and its derivatives belong to the group of macrocyclic resorcylic acid lactones and are typically formed by molds of the genus *Fusarium* (e.g., the species *F. graminearum*, *F. moniliforme* and *F. culmorum*) [29,30]. Adverse effects of ZEN in farm animals were extensively described [31–33]. The most frequently described property of ZEN is its estrogenicity. Pigs are most susceptible to those effects [30,34]. In addition, genotoxic, carcinogenic and immunotoxic effects of ZEN were reported [30] Therefore, the European Commission published guidance values for ZEN in feed that amount to 100, 250 and 500 µg/kg for piglets, sows and calves/dairy cattle, respectively [35,36]. These values were exceeded in some samples of maize destined to be used as animal feed [37–39].

Since both mycoestrogens and phytoestrogens can occur simultaneously in feed [40], combinatory estrogenic effects might be possible. Vejdovszky et al. showed estrogenic effects of ZEN and GEN depending on the used ratios and concentrations in vitro [41]. However, further data on the combinatory effects of phyto- and mycoestrogens are lacking.

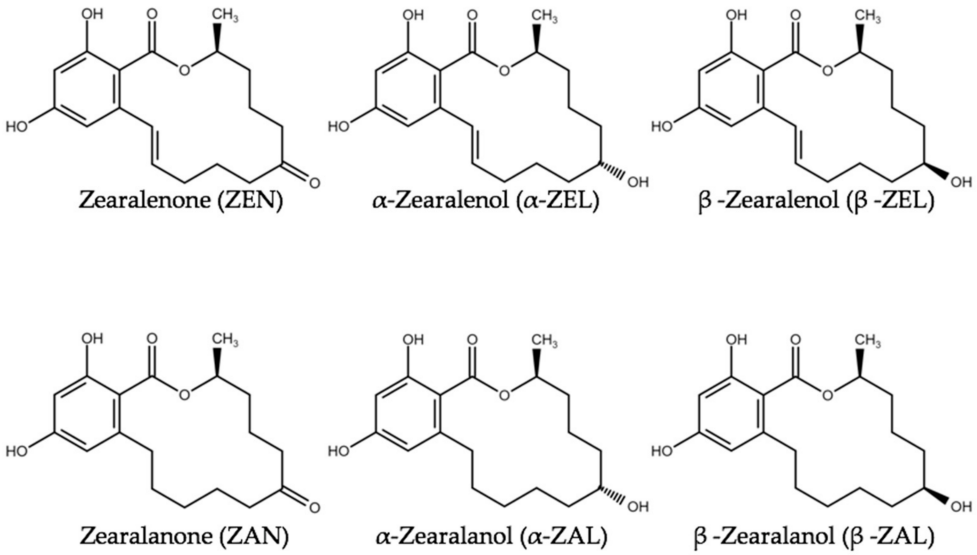


**Figure 2.** Structure of the mycotoxin zearalenone and its phase I metabolites.

This current review summarizes the metabolization of ISF including the influence of the microbiome, phase I and phase II metabolites as well as the distribution and inter-individual variability. Furthermore, in vivo studies on effects of ISF in farm animals are reviewed. The focus is on pigs, poultry, and ruminants. In addition, effects in fish are summarized briefly. Furthermore, published data on the occurrence of ISF in feed and co-occurrence with mycoestrogens are presented and supplemented by occurrence data obtained during our own studies.

## 2. Metabolism of ISF

### *2.1. Influence of the Microbiota*

The intestinal microbiome plays an important role in the digestion of various food components. Bacteria that colonize the digestive tract are known to modify ISF, which are represented in plants as both glycosides and aglycones. Before ISF can be absorbed from the gut, the sugars of the glycosides must be deconjugated by β-glucosidases expressed by intestinal bacteria and subsequently, ISF enter the bloodstream via passive absorption [42–44]. Mammalian β-glucosidase activity does not appear to substantially contribute to deconjugation of ISF glycosides in monogastric animals due to its lower expression level [45].

Apart from the deglycosylation of ISF, further metabolic conversions of ISF are catalyzed by the gut microbiome. In vitro, metabolism of GLY by monogastric fecal flora starts with demethylation to 6-hydroxy-DAI, followed by reduction to 6-hydroxy-dihydro-DAI and subsequent metabolization either by cleavage of the C-ring to 5′-hydroxy-*O*-demethyl-angolensin or by maintenance of the C-ring to 6-hydroxy-equol [46].

Biochanin A can be demethylated to GEN by the gut microbiome, both in ruminants and in monogastric animals. GEN is then further processed by the intestinal microflora in different ways depending on the animal species [47,48]. In ruminants, GEN is degraded to *p*-ethyl phenol and organic acids by ring opening [48]. In monogastric animals, GEN is reduced to dihydro-GEN by the intestinal microflora and further metabolized to 6-hydroxyif-*O*-demethyl-angolensin (6-OH-ODMA) [47].

Analogous to biochanin A, formononetin is demethylated to DAI and subsequently reduced to dihydro-DAI (DHD), which is then either degraded by cleavage of the C-ring to *O*-demethyl-angolensin (ODMA) or the formation of equol (EQ) is initiated while retaining the C-ring. This metabolic degradation of DAI is observed in all livestock species [48–50]. However, the capacity of EQ formation varies between the different species. Ruminants possess gut microbiota that favor the biosynthesis of EQ and therefore all ruminants are

considered EQ producers [51,52], whereas in pigs the capacity to form EQ is limited and only pigs with certain bacterial strains are classified as EQ producers [53].

The different ability to produce EQ between species is due to the dissimilar composition of the gut microbiota and can also vary with age. The metabolic conversion of DAI to DHD and further to EQ was observed by bacterial strains SNU NiuO16 and SNU Julong732 isolated from bovine rumen [54]. In addition, bacterial strain *Slackia* sp. D-G6 isolated from chicken intestines was described to produce EQ [55]. Similarly, *Eubacterium* strains D1 and D2 isolated from pig feces also possess the ability of this metabolic conversion of DAI. However, these isolated strains showed a lower metabolic conversion to EQ than complex mixtures of different fecal bacteria, indicating that other bacterial species are also involved in the formation of EQ [56].

### *2.2. Phase I Metabolism*

After absorption, ISF undergo further metabolic processes in the intestine and liver. During phase I metabolism, oxidative modifications by cytochrome P450-dependent monooxygenases (CYP) occur. In vitro, GEN and DAI are converted by rat liver microsomes to different mono-, di-, and trihydroxylated compounds [57]. Biliary and intestinal CYP isozymes (e.g., 1A1, 1A2, 1B1, 2E1 and 3A4) involved in hydroxylation of GEN were already identified in the late 1990s [58]. A similar metabolic pattern was shown for EQ in microsomes of the same species. EQ was metabolized to mono- and dihydroxylated compounds, with 3′- and 8-hydroxyequol being the major products [59]. In a similar experiment, GLY was mainly transformed in rat liver microsomes to two monohydroxylated GLY derivatives and the demethylation product 6-OH-DAI. These results are supported by in vivo studies in rats [46]. In liver microsomes isolated out of sheep and cattle, the conversion of formononetin to DAI was found to be very low. Further conversion to EQ could not be induced by these microsomes [60]. Studies with microsomes of other farm animals are currently unavailable.

The oxidative metabolites of ISF formed in the liver may undergo enterohepatic circulation and therefore, further metabolic conversion by the gut microbiota could be initiated as shown for EQ [61]. Due to their pyrogallol and catechol structure, the oxidative metabolites show a lower stability than their precursors and are therefore difficult to quantify. For humans, it was estimated that less than 10% of the total ISF content is present as oxidative metabolites in urine [62], whereas bacterial metabolites are of greater importance [63].

### *2.3. Phase II Metabolism*

In the further process, ISF are converted to their glucuronides and sulfates in the intestine and liver by means of enzymatic conversion. SULT1A1, among other sulfotransferase (SULT) isoenzymes, is responsible for sulfation, and isoenzymes of uridine diphosphate (UDP)-glucuronosyltransferase (UGT) accomplish the conjugation with glucuronic acid [53]. These conjugation reactions are considered to be the main detoxification pathway for ISF. Cheetahs, for example, which cannot conjugate ISF, showed symptoms of liver damage and infertility after a soy-containing diet [64].

Conjugation occurs mainly at the hydroxy group at C-7 [65–67]. Predominantly monoglucuronides, but also diglucuronides, mono- and disulfates, and sulfoglucuronides were reported [53]. However, the pattern of phase II metabolites differed not only between species, but also between genders. In humans, the main conjugate of phase II metabolism for DAI was the 7-glucuronide-4′-sulfate. For GEN, the metabolites 7-glucuronide-4′-sulfate as well as the 7,4′-diglucuronide predominate. However, differences between sex were not observed in humans. Rats, on the other hand, showed a divergent phase II metabolite profile between genders. Female rats displayed preferred production of 7-glucuronides for both GEN and DAI. The male counterpart exhibited primarily the production of 7,4′-disulfate and 7-glucuronide-4′-sulfate [68]. A possible explanation for the prioritizing formation of sulfates in male rats can be explained by elevated SULT1A1 mRNA levels [69].

Conjugation with sulfates and glucuronic acid occurred predominantly in the liver, but may also involve the gastrointestinal tract [70]. The gastrointestinal epithelium represents an important location for detoxification of phytoestrogens in ruminants. However, there are also differences between species. The conjugation activity of the ISF formononetin, DAI and EQ was up to 20 times higher in sheep compared to cows in almost all parts of the gastrointestinal tract. Among the three ISF mentioned, EQ showed the highest conjugative activity for both species [71].

### *2.4. Distribution*

Following absorption and metabolization in both the intestine and liver, ISF are transferred to various body fluids and tissues. These include plasma, urine, feces, and milk on the one hand and kidney, liver, ovary, uterus to name a few on the other hand [53,72–74]. In addition, there is evidence that ISF can cross the blood-brain barrier as well as the placenta [75,76]. Depending on the animal species, ISF and their metabolites are present more or less conjugated in plasma and urine. Although in pigs the proportion of aglycones in these fluids is usually less than 5%, the proportion of non-conjugated ISF in urine may be up to 50% in rats and up to 90% in monkeys [53]. ISF are also found in tissues in different forms depending on the animal species. For example, in rats the ISF are predominantly present as aglycones, whereas in sheep the vast majority of ISF were detected in conjugated form [73,77].

### *2.5. Inter-Individual Variability*

Several different factors may influence biokinetics and bioavailability of ISF such as intestinal microflora, age, composition of feed, and duration of soy consumption. Contradictory data are available on the concentration of EQ in the blood of pigs. Although one study did not detect EQ in the serum of piglets (age 30 days) [53], another study detected EQ in serum of pigs (age 183 days) [78]. This observation might be explained by the different age of the investigated animals. This phenomenon was also observed in rats. Although older rats can produce EQ, no EQ was detected in 3 months old rats [79]. This is attributed to the presence or absence of certain bacterial species that establish itself in the gut in the course of a lifetime [79].

The composition of the feed also seems to have an influence on the metabolism and bioavailability of ISF. It appears that a higher proportion of carbohydrates in the diet and a reduction in fat favors EQ production [80]. Higher levels of carbohydrates may stimulate fermentation in vitro and contribute to increased EQ formation [81]. Likewise, an increase in bioavailability may occur when the feed consists to a minor percentage of oligo-fructose as has been the case in rats for DAI and GEN [82]. In addition, genetic polymorphisms of xenobiotic-metabolizing enzymes such as CYP and UGT may affect bioavailability and metabolism. The genotypes (Val/Leu) and (Leu/Leu) of the gene *CYP1B1* are suspected to contribute to an increased risk of breast cancer at a low energy-adjusted daily soy ISF intake [83]. Likewise, the gene polymorphism *UGT1a1*28*, which is responsible for the conjugation of xenobiotics, has an influence on the metabolic pattern. The presence of this polymorphism in individuals leads to increased excretion of GLY-, DAI-, and GEN-sulfates, causing a decreased production and urinary excretion of glucuronides [84].

### *2.6. In Vivo*

#### 2.6.1. Ruminants

Compared to monogastric animals, the ruminant's stomach works in a completely different way. Therefore, it is not surprising that also the metabolization of phytoestrogens is different (Figure 3). The rumen is the primary location of the deglycosylation of the ISF glucosides mainly present in the plants, and other transformation processes of the aglycones [48]. Biochanin A is metabolized by demethylation to GEN and further by ring cleavage to para-ethylphenol and organic acids [85]. Formononetin is predominantly demethylated to DAI [85]. EQ is formed from DAI by hydrogenation and ring cleavage.

Contrary to humans and pigs, ruminants are principally EQ producers [85]. The metabolites formed from biochanin A and GEN are estrogen-inactive substances. Metabolism of formononetin, however, leads to formation of the more estrogenic metabolite EQ [86]. The metabolic processes catalyzed by microorganisms in the rumen may last six to ten days after ingestion [87]. In cows, an ISF-rich diet can play an essential role in their metabolism. One study investigated the degradation of dietary ISF in rumen fluid from cows fed with either a hay diet or a concentrate-rich diet, both including 40% soybean extract. The results showed faster metabolism of both DAI and GEN under concentrate-rich conditions and an overall higher production of EQ under the hay diet conditions [88]. No significant differences were observed in GLY degradation between the two diet conditions. However, using higher amounts of soybean extracts up to 75 mg per 40 mL rumen fluid resulted in a decrease in EQ production, most likely because of the inhibitory effects of GEN on the rumen microflora [88]. This decrease is in agreement with a previous study [50]. Only a minimal percentage of hydrolyzed phytoestrogens is absorbed directly from the rumen into the bloodstream [85]. The majority is first subject to further conjugation, predominantly with glucuronic acid [85]. This already takes place in the gastrointestinal epithelium and only a tiny percentage is conjugated in the liver [85]. This fact suggests that in ruminants the liver plays a minor role as an organ for ISF detoxification [85].

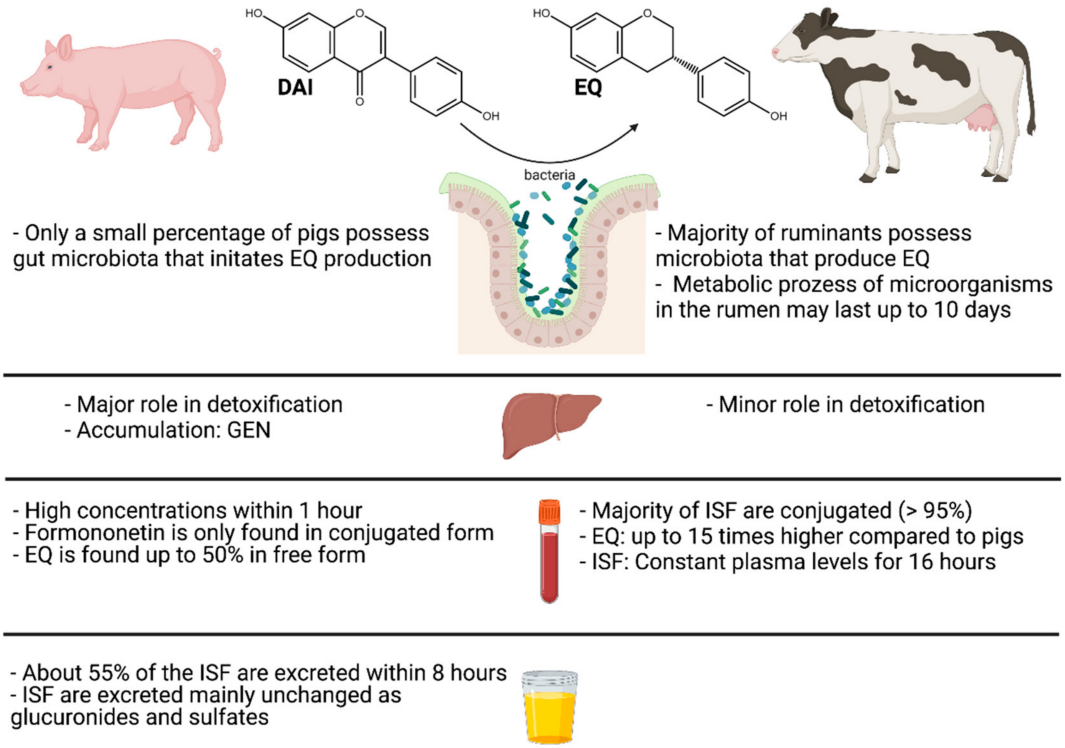


**Figure 3.** Comparison of the metabolic pattern of ISF between pigs (**left**) and ruminants (**right**). ISF = isoflavone(s); DAI = daidzein, EQ = equol; GEN = genistein. To the best of our knowledge no data are available concerning the excretion of ISF in ruminants. Figure created with BioRender.com.

Feeding trials performed in dairy cattle and ewes revealed that the absorption and distribution of ISF differs within these two species. Formononetin (530 mg/kg feed) and DAI (12.6 mg/kg feed) were absorbed very rapidly in dairy cattle, reaching concentrations of about 90 and 50 µg/L in blood plasma, respectively [51]. The concentration of these two ISF was three times higher within the first hour compared to the concentration detected in sheep when fed with the same ISF concentration [51]. The EQ concentration in dairy cows was maintained at a constant level of about 180 µg per 100 mL plasma over a 16-h period. Initially, sheep showed a lower total (free and conjugated) plasma EQ concentration,

which increased after three hours to the same level as in cows. However, after 16 h, the EQ concentration was half as high as in dairy cows, indicating a faster metabolism of ISF in sheep [51,85]. The amount of unconjugated ISF in the blood was ≤5% for either species, whereby the concentration of free EQ was 10-fold higher in dairy cows compared to ewes at all time points. Nevertheless, sheep are described to be more sensitive to EQ [85]. One of the most plausible explanations is that the estrogen receptors in the uterus are expressed 2–4 times more in sheep than in cows [89,90]. Considering that the relative estrogenic potency of EQ is 0.061% of 17-β-estradiol [91] and that free EQ in sheep can reach concentrations of 20 ng per 100 mL, EQ could reach a 100-fold higher estrogenic potency than 17-β-estradiol during estrus [92].

#### 2.6.2. Pigs

The metabolic profile of ISF in pigs was investigated in a feeding trial with diets containing 20% red clover with previously determined levels of ISF, corresponding to an average daily intake of 97 mg GEN, 88 mg DAI, 866 mg formononetin and 378 mg biochanin A [85]. Using a permanent vein catheter, the concentrations of the above-mentioned ISF could be determined at different time points after feed intake. Within one hour the total (free and conjugated) maximum level of formononetin (100–120 µg/100 mL plasma) was 10 times higher than the level detected in bovine plasma that was also investigated in this study. This observation that formononetin can be detected very rapidly in blood plasma suggests that it is already absorbed to a large extent in the stomach. DAI (5–7 µg/100 mL plasma) and EQ (12–25 µg/100 mL plasma) likewise exhibited the maximum level detected within one hour of feed ingestion, although at 5–15 times lower concentrations compared to formononetin [85]. Other feeding trials have shown that depending on the feed composition, different ISF are primarily detectable in plasma. Measurements after feeding of a soybean meal (SBM) on the one hand and soy protein concentrate (SPC) on the other hand showed that after hydrolytic cleavage of phase II metabolites such as glucuronides and sulfates of ISF, DAI and GEN were detected in higher amounts in the plasma of SBM fed animals [93].

Formononetin was almost exclusively found in the conjugated form, whereas EQ was found up to 50% in free form in plasma [85]. However, the total amount of EQ in pigs is up to 15 times lower compared to other species such as ruminants [85]. The different ability to produce EQ between species is largely due to the different composition of the gut microbiota. Thus, it appears that pigs in comparison to ruminants possess only a small percentage of the gut microbiota that initiates the conversion to EQ [53]. However, the fact that EQ is detectable in blood and tissues of pigs strongly suggests that pigs are also EQ producers [78,93]. Although Lundh et al. found EQ as a major metabolite of soybean and red clover ISF in pig plasma, Gu et al. detected no EQ in pig serum [53,85]. The difference between these observations might be due to the age difference. In young piglets the intestinal microflora might not be developed sufficiently to produce EQ.

The major metabolites recovered in mammary tissue of pigs were DAI and EQ [78]. These two ISF exhibit estrogenic activity, and both may accumulate in tissues of the reproductive tract and thus strongly affect the reproductive system in pigs [78]. In contrast to DAI and EQ, GEN was mainly detected in the liver of pigs [78]. After absorption, ISF are converted to their glucuronides and sulfates in the intestine and liver by enzymatic conversion [53]. Sulfate conjugates are thought to have a higher estrogenic potential compared to glucuronides [94,95].

About 55% of the ISF were excreted via urine within the first 8 h after feed intake [85]. In the case of formononetin, 72% was excreted via urine without metabolic changes [85]. GEN was also excreted mainly unchanged via urine and only to a minor percentage the metabolite dihydro-GEN was detected [53]. In addition to DAI as the main component of urinary excretion, the metabolites *O*-demethyl-angolensin (ODMA), EQ, and DHD were determined [53].

#### 2.6.3. Poultry

Several in vitro and in vivo studies on the effects of ISF have been conducted in poultry, especially chickens. Soybean ISF constitute a large part of their diet, and these phytoestrogens transfer to and accumulate in the tissues and eggs of hens [96,97].

In 2001, an experiment conducted at the Tokyo University of Agriculture revealed the transfer of soy ISF to the plasma and egg yolk of laying hens. A group of 5 laying hens was fed a high dietary concentration of soy ISF extracted from soybean hypocotyls over an 18-day period. The concentration of ISF in the ISF-enriched diet was at least 3 times higher than the normal diet, containing for the enriched diet 353 mg/kg, 26.2 mg/kg and 476.9 mg/kg of DAI, GLY, and GEN, respectively. Analytical data showed a sharp increase in the concentrations of total ISF in plasma and egg yolk until day 12 of feeding with values of 3.2 nmol/L in plasma and 65.3 µg/100 g in egg yolk [96]. Another study reported the transformation of DAI to EQ which then accumulated in the egg yolk of laying hens. The results showed that although most of the ISF (DAI, GLY, GEN) were present in blood and yolk, the concentration of EQ was much higher than the concentrations of other ISF, especially in the egg yolk [97].

## 3. Effects of ISF

### *3.1. Ruminants*

Several studies have been conducted in ruminants such as cows and sheep reporting both positive and negative effects on the health of these animals. Several studies revealed the excretion of ingested ISF in ruminant's milk and tissues, as well as increased growth and reduced fertility upon ISF exposure [21–24].

A study investigated the effects of ISF-enriched feed on the carry-over of ISF to milk and on the rumen microbiota in lactating Czech Fleckvieh x Holstein cows. The experimental group received a basal diet supplemented with 40% more soybean ISF extract (16,006 mg/day) compared to the control group (8401 mg/day). As a result, the concentration of EQ in milk was nearly 2.5 times higher in the experimental group. Additionally, the experimental cows had a reduction in microbial richness compared to control cows [22]. Interestingly, several publications report a high concentration of EQ in bovine milk. A study in Denmark reported a very high EQ concentration of 230 µg/L in organic milk [98]. An even higher EQ concentration of 293 µg/L was detected in the milk of Australian cows that received a high clover diet [99]. Similar to soybean, red clover (*Trifolium pretense* L.) contains phytoestrogens with the prominent ones being formononetin and biochanin A [100]. It plays a vital role in agricultural processes and has a high crude protein content and fiber, making it an important and feasible option for feeding ruminants. Dairy cows fed with red clover silage have increased milk production as well as higher intake of nutrients compared to animals fed green silage [23,24,101,102]. Similar to the ISF measurements in Australian and Danish bovine milk, two studies performed in France found EQ concentrations of up to 191 µg/L and 1120 µg/L in cow and goat milk, respectively [103,104]. Though DAI and GEN were also present in milk, their concentrations were lower compared to EQ concentration [99,103,104]. Furthermore, an EQ concentration of 364 µg/L in cows fed with red and white clover silage was also reported [9]. These results are in line with another study where cows were fed with a similar diet [105,106].

The concentration of EQ has been studied not only in milk, but also in meat and tissues [73,107]. In ewes fed a diet of red clover, EQ and DAI found as glucuronides reached high concentrations in plasma. However, these compounds were not equally distributed in different tissues with the highest amount found in the kidney [73]. Lambs fed with red clover pastures showed higher weight gain and fiber intake compared to those receiving a ISF free diet [19,20,108]. These observations were in line with another study where red clover-fed lambs had gained more weight than those fed a white clover pasture [109]. Another supporting trial also reported faster growth rates of lambs fed with red clover silage [110].

Though phytoestrogens can have a positive effect on milk production of cattle, they can also lead to adverse effects on the reproductive system of both cows and sheep. Several studies have been carried out to test the effects of ISF on the reproduction rate and reproductive tract of the animals, and varying results have been observed in both species [87]. A series of pathological examinations in animals fed mainly with red clover showed signs of cervicitis and hyperplasia in the endometrium of sheep [87]. Chronic infertility was observed in sheep after continuous exposure to ISF, reflected by symptoms that included irreversible changes in estrogen-sensitive organs and completely masculinized ewes [87]. In cattle, only temporary infertility has been observed so far due to ISF exposure. This is reflected by a reduced conception and ovulation rate [87]. In addition, other symptoms such as irregular estrus cycles, vulvar swelling, cyst formation and behavioral abnormalities such as absence of estrus and nymphomania were reported [87]. After discontinuation of ISF exposure, symptoms decreased over a few days to weeks and disappeared with time [87]. Cattle are less sensitive to exposure to ISF compared to other ruminants and this is suspected to be due to differences in estrogen receptors and tremendous weight differences [51]. Formononetin and DAI, which are converted in the rumen into the highly estrogenic EQ, are mainly responsible for infertility [111]. Only three out of five cows fed a soybean-rich diet became pregnant after successful insemination, compared to four out of five cows in the control group [112]. Furthermore, in the same study, cows that received a soybean-rich diet showed elevated levels of prostaglandin $F_{2\alpha}$ in the blood that were positively correlated with EQ concentrations. The ISF GEN and biochanin A have also been associated with infertility [113]. In one case study, a red clover silage diet caused vaginal discharge and irregular estrous cycles leading to higher miscarriages and premature conception rates in cows [114]. These changes were reversible when feeding of red clover was discontinued [114]. In agreement with these observations, other studies reported the onset of vaginal prolapses, infertility, increased udder size and growth of ovarian cysts in cows fed with red or subterranean clover [115]. Furthermore, cows that received a soy-containing diet showed a lower pregnancy rate and needed a higher number of artificial inseminations than cows that received soy-free diet [112]. In contrast, one study described increased fertility of heifers fed red clover silage [116]. In this study, the heifers were fed either red clover or grass silage prior to and during the insemination period. Cows that received the red clover silage diet showed a significantly higher pregnancy rate to first service, with an increase of 33% [116].

Infertility of sheep fed with a clover diet gave rise to the so-called "clover disease" [28]. The disease can be classified according to certain clinical conditions such as infertility in ewes that goes hand in hand with changes in the endometrium, prolapse of uterus in unmated ewes and the death of lambs during delivery due to failure of the cervix to dilate properly, mammary development and lactation in unmated ewes and wethers (castrated male sheep), blockage of urethra and death in wethers [21]. Furthermore, sheep that were fed a clover-rich diet showed a loss and or reduction in reproduction [117]. The effects of ISF can cause two different types of infertility problems in sheep, either homeostatic reproduction was recovered after the absence of clover diet or infertility worsening with continued clover-feeding [21]. Indefinite and irreversible infertility in ewes is characterized by a decrease in pregnancy rates, secretory function changes of the cervix and loss of the mucous membrane [21,118,119]. Late pregnant ewes that received clover silage from two months prepartum until estrus induction showed a higher interval to estrus, shorter estrus duration and tended to show a decreased litter size compared to ewes that received maize silage [120]. Red clover silage with a high phytoestrogen content fed to nulliparous ewes before, during and after the breeding season did not reduce fecundity compared to a control group that received timothy/meadow fescue grass silage. However, the volume of fetal fluids increased in ewes that received red clover silage, which could increase the risk of vaginal prolapse before the term [121].

In Sweden, a study investigating the effects of dietary phytoestrogens on plasma testosterone and triiodothyronine (T3) levels in male goats was conducted [122]. From

3 months of age to 6 months of age, the goats received either a basal diet or a diet rich in phytoestrogens. In the first 7 weeks, no significant difference in the concentration of plasma testosterone was observed between the two groups. By the fifth month, the goats fed the ISF-rich diet showed significantly higher testosterone concentrations compared to the control animals. Additionally, the concentration of free T3 was also higher in animals that received ISF-rich feed. These findings suggest that ISF can promote testosterone synthesis during the puberty stage of male goats by increasing secretion of T3 [122].

Concluding, in ruminants, different effects of ISF were reported depending on the animal species and the study design. Cows displayed an increase in metabolism and milk production when they received ISF-rich feed. Animals fed clover silage especially showed increased milk production and nutrient intake [23,88,101,102]. A high concentration of EQ, DAI, and GEN was found in the milk of cows, as well as EQ and DAI in the plasma, meat, and tissue of sheep [73,105,107,123]. Similar to cows, lambs fed with red clover pastures (ISF-rich diet) reported higher weight gain and fiber intake, contributing to a faster growth rate of these animals [109]. Unlike poultry, ISF led to an adverse effect on the reproductive system of ruminants, where they caused either a temporary or permanent form of infertility in sheep. The effects of ISF on the reproduction and fertility rate of cows was not as profound, with a few reported miscarriages and infertility which was reversible [51,87]. Additionally, in male goats, ISF promoted the onset of puberty by increasing testosterone synthesis [122]. Since soy is one of the most widely used protein sources, an adverse effect on the reproductive system due to the high content of ISF cannot be excluded. In fact, an influence of soy-rich diets on female reproductivity in cows can be observed.

### *3.2. Pigs*

Phytoestrogens may affect the growth, meat quality, and immune response system of pigs and are therefore of great interest for the swine agriculture industry. There are several studies on the effect of soybean meal on the growth, intestinal morphology, and antioxidative properties in pigs and piglets. In one experiment, groups of piglets were fed different types of feed, i.e., corn-soybean meal (C-SBM), corn-soy protein concentrate (low ISF C-SPC) or C-SPC enriched with the same ISF concentration as in C-SBM over a period of 72 days [25]. Piglets fed the C-SBM and C-SPC + ISF diets showed higher body weight and greater villus height on day 72 than piglets that received C-SPC feed. In comparison to the C-SPC diet, the pigs fed with dietary C-SPC + ISF displayed an increase in plasma superoxide dismutase (SOD) activity on days 28 and 42. This was also followed by a reduction in plasma malondialdehyde content on day 42. In conclusion, the feeding of soybean ISF over a long period of time improved growth performance and antioxidative activity, as well as protection of the intestinal morphology [25]. Lipopolysaccharide (LPS)-induced retardation in growth performance, diarrhea, and high plasma concentrations of endotoxins and malondialdehyde have been reversed by supplementation with ISF [124]. Furthermore, LPS challenge significantly increased the abundance of p-p38 and TRL4 proteins in the jejunal mucosa of piglets which are thought to be involved in intestinal damage. The addition of ISF resulted in a decrease of these proteins [124]. A case study determining the effects of dietary soy ISF on growth, carcass traits and meat quality in nearly fully grown pigs was performed [125]. Pigs were allocated to three different diets: (1) C-SBM; (2) C-SPC or (3) C-SPC + ISF. Pigs fed the C-SPC + ISF diet showed an increase in carcass length, percentage ham lean and thaw loss, combined with an overall reduction in total ham fat [125]. Pregnant sows that received dietary DAI and GLY from day 85 of gestation littered piglets with higher birth weight and average daily gain [18,126]. However, the effect of ISF on growth performance is contradictory in the literature. In a study by Xiao and coworkers, piglets that received a dietary DAI level of 400 mg/kg showed lower average daily weight gain than the control group that did not receive DAI [15]. The discrepancy between the observed effects of ISF on growth performance may be due to differences in age of the animals, duration of the study or the kind and dose of

ISF. Furthermore, such high DAI concentration had a trend to cause mild damage of the kidneys, livers and spleens of the weanling pigs after 70 days [15].

Several studies have reported beneficial effects of ISF in pigs challenged with viral infections. One study investigated the effects of dietary SBM infused with ISF on the growth performance and immune response of pigs inoculated and infected with porcine reproductive and respiratory syndrome virus (PRRSV). The effect of ISF enhanced immune response by reduced concentrations of serum viral load and improved growth [127]. This agreed with another study about the effects of dietary soy ISF on the response of weanling pigs to PRRSV. The ISF supplemented to the pigs reduced PRRSV-induced circulating neutrophils and improved the cytotoxic-to-helper T-cell ratios [128]. The findings suggest that ISF contribute to activating the adaptive immune system and in clearance of viral infections [128]. Furthermore, the effect of dietary soy GEN on pig growth and viral replication was also investigated. Increasing concentrations of dietary GEN were associated with a decrease in serum content of the PRRS virus and a quadratic increase in daily feed intake [129]. Furthermore, a study by Smith and coworkers found that dietary ISF reduced the mortality of pigs challenged with PRRSV [130].

One study investigated the effects of a high DAI dose (640 mg/kg feed) on the redox system in tissues of finishing pigs over a period of 64 days [16]. On the one hand, the supplemented high dosage increased SOD activity and total antioxidant capacity in the longissimus muscle of the animals, and it reduced activity of NADPH oxidase-2 and cyclooxygenase-2, enzymes involved in the production of reactive oxygen species. However, on the other hand, it also induced pro-oxidant changes in back and abdominal fat, liver, and plasma tissues by promoting the expression of reactive oxygen species producing enzymes. To conclude, finishing pigs fed with high doses of DAI show improved redox activity in muscle and, in contrast, increased oxidation levels in liver and fat tissues [16]. Similar to its precursor DAI, the metabolite EQ also displays antioxidant effects in piglets. A case study reported that lactulose increased EQ production and improved liver antioxidant status in barrows supplemented with DAI [131]. After 20 days, EQ concentrations were significantly elevated in both urine and fecal samples. Additionally, the activity of the enzymes SOD and copper-zinc SOD increased in the livers of barrows, thus improving the overall antioxidant characteristics of the animals [131].

The effects of ISF on the reproductive system of pigs have also been studied, though not as much as in sheep and cattle. A study from China investigated the effects of soybean ISF on male reproductive parameters using Chinese mini-pig boars as a model [132]. Dietary concentrations of soybean ISF of 0 to 500 mg/kg were administered to the pigs for 60 days. An ISF concentration of 250 mg/kg increased the testis index, fructose content in testicular tissue, alpha-glycosidase content in testicular tissue, as well as viable germ cells and level of apoptosis regulating Bcl-2 protein in testicular tissue. Pigs fed 500 mg/kg of soybean ISF exhibited a significant decrease in testis and epididymis indexes and serum testosterone levels, as well as an increase in numbers of early and late apoptotic germ cells and level of pro-apoptotic BAX proteins in the testis. In conclusion, low to moderate consumption of soybean ISF did not affect reproductive parameters in the mini-pig board, whereas higher concentrations negatively affected male reproductive health [132].

Similar to the Chinese mini-pig boars, another study also investigated the effects of soybean ISF on the onset of puberty, serum hormone concentration, and gene expression in hypothalamus, pituitary gland and ovary of female Bama miniature pigs [133]. The pigs received dietary soybean ISF doses between 0 and 1250 mg/kg. Compared to the control group, the pigs fed 1250 mg/kg soybean ISF exhibited a significant delay in the onset of puberty. At 4 months of age, a reduction in serum concentration was observed for gonadotrophin releasing hormone and luteinizing hormone, whereas the serum concentration of follicle-stimulating hormone increased. The soybean ISF-supplemented pigs also showed a reduction in ovary of steroidogenic acute regulatory protein and KiSS-1 metastasis-suppressor. To conclude, soybean ISF adversely affected the reproduction sys-

tem in female pigs by delaying the onset of puberty due to the decreased expression in reproduction-regulating genes [133].

Feeding red clover silage to pigs induced signs of hyperestrogenism such as fertility problems [85]. Further feeding trials of 10- to 24-week-old piglets with soy showed that the soy-containing diet induced estrogenic effects in the piglets [134]. Again, symptoms of hyperestrogenism such as swelling of the mammary gland and vulva, enlargement of the uterus, and pathological changes of ovaries were observed. These data also suggest that with continuous feeding of soy, the vulva size increased with each week. This observation implies that there is an enhanced estrogenic effect over time due to cumulative effects [134].

Ovariectomized gilts are often used as a model to study estrogenic properties of different substances. Because they have a deficit in estrogens, the effect of mildly estrogenic substances can be determined. Therefore, Ford and coworkers [135] studied the effect of GEN in ovariectomized gilts. The gilts received daily intramuscular injections of 50–400 mg GEN. Doses of $\geq$200 mg caused a significant increase in cervix and uterine mass.

DAI exposure of pregnant sows caused swelling and reddening of the vulva in neonatal piglets [136]. Compared to the control group, the male newborn piglets whose mothers were fed with DAI had a significantly higher birth weight. No other influences on the piglets were observed. However, the addition of GEN and DAI to the feed of lactating sows caused estrogenic effects in the piglets, which are most susceptible to exogenous endocrine disruptive chemicals (EDC) at this stage of their lives. It can be concluded that ISF exposure through breast milk led to an exposure of the estrogenic substances to the piglets causing hyperestrogenism in female piglets [136].

In conclusion, ISF were shown to exert both positive and negative effects in pigs. Soybean meal improved the growth performance and antioxidative activity in pigs [15,124–126]. In PRRSV-challenged pigs, ISF improved immune functions and reduced mortality [129,131]. However, phytoestrogens also showed negative effects. ISF caused hyperestrogenism in female piglets [85,134]. Similar to what was observed in sheep, high consumption of soybean ISF negatively affected male reproduction [132]. Furthermore, high doses of ISF were reported to have toxic effects including increased oxidation levels in liver and fat tissues [16] and soybean meal fed pigs showed reduced total ham fat [124].

### *3.3. Poultry*

Chicken that were fed with a soy ISF-rich diet (starting at 1000 mg ISF/kg feed) showed significantly higher plasma levels of estradiol compared to chicken on a basal diet. The same chicken were also much healthier as shown by improved egg and white egg weight over the course of the experimental period of 3 months [17]. The improvement in egg weight is in agreement with increased egg production and quality, as reported in previous studies [96,137]. Studies investigating the effects of soy ISF (DAI, GEN, GLY) on the laying performance of Xueshan breeder hens found that an ISF diet increased yolk color and serum total antioxidant capacity and immune levels [138–140]. Furthermore, ISF caused an increase in hatching rate potential of 36-weeks old hens [138]. However, ISF caused a reduction in average egg weight in 52-weeks old hens and a decrease in egg rate in 44-weeks old hens [138]. Dietary administration of DAI to laying hens at doses of 10, 100 or 200 mg/kg for 12 weeks increased hatchability [139]. Furthermore, dietary DAI doses of 10, 20 and 30 mg/kg showed a positive effect on egg weight and fertility in Zhedong white geese [141].

Ni and coworkers investigated the effect of DAI on the egg-laying performance in Shaoxing duck breeders [137]. The following criteria were measured: egg-laying rate, egg composition, feed conversion ratio, hatchability characteristics of eggs and body weight, ovary and oviduct weight, and changes in serum concentrations. Doses of 3 mg/kg and 5 mg/kg DAI administered to ducks over 35 days resulted in an increase in the egg-laying rate, mean egg weight, and feed conversion ratio. However, a negative effect on fertility and hatchability responses was observed. Extending the feeding period to 63 days caused a 7.7% increase in the egg-laying rate, as well as higher body and oviduct weight.

Additionally, the yolk and albumen ratio were reduced. This suggested that DAI has ambiguous effects on the laying performance of the ducks depending on the physiological conditions and DAI dose [142]. The impact on the laying rate seems to depend on the age of poultry. In 7-months old female quails, supplementation of 6 mg/kg DAI led to a decrease in laying rate, whereas the same amount of DAI led to an increase in laying rate in 12 months old quails [13].

Different effects have been observed in Japanese quails. In one study, ISF administered to female quails via the diet did not affect growth, feed intake or weight of oviduct [14]. Likewise, ISF did not affect growth or feed intake in male quails in the same study. However, the same male quails exhibited a 40% reduction in photoperiod-induced testis development suggesting ISF negatively affect the reproduction system of male quails [14]. It was reported that the supplementation of GEN in Japanese quail suppressed spontaneous oviduct tumorigenesis. The results indicated that GEN supplementation significantly reduced the occurrence and size of spontaneously occurring leiomyoma of the oviduct in the quail [143].

In a study in broiler chickens very high dietary concentrations of soy ISF (starting at 693 mg/kg) decreased the growth rate [144]. In contrast, the administration of lower ISF concentrations (10 and 20 mg/kg) increased the weight and feed intake of male broilers compared to the control group [145]. Following, one study could show that supplementation of GEN (400 mg/kg) fed for 8 weeks significantly improved the reproductive activity and bone status of laying broiler breeder hens. The GEN-rich diet induced an increase in the levels of vitellogenin, progesterone, and follicle-stimulating hormone in the serum [146]. In addition, the levels of malonaldehyde in the follicle and egg yolk of hens decreased, while calcium and phosphorus levels increased in the tibia, which explains the improved strength of tibia bone [146].

Antioxidant effects of ISF have also been observed in poultry. One study highlighted the positive benefits of an ISF-rich diet on broiler chickens suffering from the infectious bursal disease virus (IBDV). Dietary ISF improved the overall health and condition of infected chickens. This effect was attributed to the decreased expression of viral protein 5 mRNA, a protein produced in response to IBDV to drive apoptosis [147,148]. ISF also decreased the onset of bursa lesions and additionally had a high antioxidant capacity [147]. Further antioxidative properties of ISF in male broiler are described by the consumption of 40 or 80 mg ISF per kg bodyweight, which leads to an increased antioxidant capability and superoxide dismutase activity in plasma [145].

To conclude, in poultry, high ISF concentrations in the diet increased the rate of egg production and hatching rate due to increased estrogen activity and weight in some studies [138,149]. However, ISF decreased average egg weight in 52-weeks old laying hens and egg rate in 44-weeks old laying hens [138]. High ISF concentrations were linked to improved antioxidant effects and immune system as well as a reduction in blood cholesterol levels, improving overall health, and such rich diets decreased the onset of bursa lesions and viral mRNA in chickens infected with infectious bursal disease virus (IBDV) [140,147,150]. Additionally, the ISF GEN affected the reproductive system and bone status of hens and ducks, by capacitating their performance and enhanced strength of their tibia bone [146]. Several negative effects of ISF were observed in quails. Dietary ISF caused a decrease in the laying rate of 7-months old female quails [13] and negatively affected the development of the reproductive tract in males [14].

### *3.4. Fish*

Studies about the effects and the metabolic fate of ISF on aquatic species are scarce. However, one research team showed that the supplementation of soy ISF to the diet of the marine fish "golden pompano" caused an increased growth rate [151]. In contrast, higher levels of ISF supplementation in diets of some other fish species such as Japanese flounder, Atlantic salmon fry and yellow perch led to a significant reduction in the weight of the animals [152–154]. Therefore, a general conclusion is not possible and in other marine species, such as striped bass fingerlings, no effect of ISF on the growth was observed [155].

These differences in the effect of ISF-supplemented diets may be due to different ISF pattern or might be species dependent. ISF supplementation is known to alter the digestive process by inducing a reduction in maltase activity, which is a key enzyme in the digestion of carbohydrates in fish [154]. In addition, a tendency towards skeletal malformation was observed in ISF-fed Atlantic salmon presumably related to the reduction of thyroid peroxidase [154]. Thyroid peroxidase is mainly involved in the synthesis of thyroid hormone (TH), which in fish plays a crucial role in the development of the musculoskeletal system [156]. Another factor that may contribute to the impaired growth of salmon fry is the depletion of glycogen in hepatocytes induced by ISF-containing diets [154].

Studies indicate that consumption of ISF leads to a decrease in whole body crude lipid content in juvenile Japanese flounder [152]. It was suggested that this decrease is due to an effect of ISF on transcription factors which modulate the expression of genes involved in lipogenesis or lipolysis [157]. Considering that there is little data on the metabolism of ISF in fish, it is also inconclusive whether fish can produce EQ or not. For example, no EQ was detected in the bile of rainbow trout fed with DAI (up to 49 mg/kg feed) [158], whereas EQ could be detected in the tissue of sturgeons (EQ intake up to 432.3 mg) [159]. However, it cannot be concluded from these results that sturgeons are in fact EQ producers as EQ was also present in feed. Additionally, the ISF profile detected in the serum indicates that sturgeons did not produce EQ in this experiment [159].

The metabolism of ISF in sturgeons seems to be delayed, as high concentrations were found in the liver (up to 3.5 mg ISF per kg liver), indicating an accumulation [159]. This accumulation can cause a chronic estrogenic effect in hepatocytes, which is reflected by increased vitellogenin synthesis observed both in vivo and in vitro [160,161]. In Siberian sturgeons, intraperitoneal administration of GEN, EQ, and biochanin A increased vitellogenin levels in blood, thus exerting an estrogenic effect [162]. Therefore, particular attention should be drawn to GEN because of its high occurrence in fish feed and accumulation in tissues, which can result in particularly high estrogenic effects [162]. In rainbow trout, GEN, DAI, and GLY can inhibit the metabolism of E2 in the kidney and liver. Inhibition of E2-metabolizing enzymes may result in increased bioavailability of E2 in peripheral target tissues. This could be another potential mechanism of how ISF induce estrogenic effects [163].

ISF had a positive effect on immune parameters in golden pompano. Feeding 40 mg/kg ISF resulted in a significant increase in C3 protein, which is part of the humoral immune response and plays a central role in the lysis of pathogenic cells and bacteria [151]. Moreover, there was an increase in plasma lysozyme activity due to ISF. Lysozyme activity is an important index of innate immunity, which plays a more important role in fish than in mammals. Numerous other immune parameters and health indicators such as increased activity of respiratory bursts, decrease of glutamic-pyruvic transaminase and oxalacetic transaminase and increase of HSP70 were induced by ISF supplementation [151].

## 4. Occurrence

### *4.1. Literature*

As pointed out in the introduction, significant concentrations of ISF are mainly found in plants of the *Fabaceae* family [164]. However, ISF content as well as the overall composition of soybeans and red clover are subject to wide variations depending on cultivar, season, and further processing [165].

In addition to contamination with ISF, contamination of feed and food with zearalenone (ZEN) and its metabolites may occur at different stages of the feed supply chain. ZEN-producing *Fusarium* species grow well in humid weather on the fruit or stalk of grain and their growth is accelerated by improper storage. Contamination with ZEN and its metabolites is common in commodities such as wheat, corn, rice, barley, soybean. [166–168].

The occurrence of ISF and the incidence of ZEN in a diverse range of feedstuffs analyzed over the last 10 years is provided in Table 1. Although there are numerous studies regarding the occurrence of ZEN and its metabolites in feed over the last decade, there

is only scarce data on the incidence of ISF. However, in all samples the detected average concentrations of ISF are in the medium to high mg/kg range. In silage samples that are composed of clover, grass, and cocksfoot, the prevalent ISF are formononetin and biochanin A and this in accordance with previous reports [100,169].

The situation is different for soybean meal and cow feed samples, where the glycosides genistin and daidzin are the predominate ISF. Cow feed samples from Thailand reached maximum concentrations of 57 and 42 mg/kg feed for genistin and daidzin, respectively [40]. The concentrations of these two ISF in soybean meal feed were even higher, reaching 1274 and 785 mg/kg feed for genistin and daidzin, respectively. No cases were reported where the aglycon showed higher concentrations than its respective glucoside [40]. Compared to the samples from the US, soybean feed samples from Brazil and Argentina had higher concentrations of ISF, which could be due to environmental factors that lead to higher concentrations of ISF in soybean meal [72]. The prevalence of ZEN was in most of the samples very high. Some samples also exceeded the EU guidance values for acceptable ZEN concentrations in pig feed [167] (EU guidance values for: piglets and gilts 0.1 mg/kg; sows and fattening pigs 0.25 mg/kg; calves and dairy cattle 500 µg/kg). Feed samples were found that exceeded the guidance value for piglets and gilts in every region of the world. For samples from East Asia, 27.3% of the analyzed samples exceeded the guidance value for piglets and gilts [167].

Among the few studies investigating the co-occurrence of ISF and ZEN or its metabolites in feed, one study from Thailand reported ZEN concentrations of 0.96–55.6 µg/kg in cow feed, and low to middle concentrations of ISF (0.030–57.9 mg/kg) in cow feed [40]. Additionally, a study published in 2021 showed that ISF co-occurred with ZEN in pasture samples from Austria [170].

**Table 1.** Occurrence data of ISF and ZEN and its metabolites in feed from literature of the last decade.

| MYT/ISF | Food Commodity | Samples Tested | Positive Samples | Country | Detection Technique | Concentration Range (µg/kg) | Average (µg/kg) | LOD (µg/kg) | LOQ (µg/kg) | Mean Recovery | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ZEN | Pig feed | 20 | 6 | China | UPLC-MS/MS | <LOQ–18.7 | 1.8 | 0.25 ng/mL | 0.75 ng/mL | >90% | [171] |
| ZEN | Cattle feed | 20 | 4 | China | UPLC-MS/MS | <LOQ–14.4 | 1.4 | 0.25 ng/mL | 0.75 ng/mL | >90% | [171] |
| ZEN | Chicken feed | 20 | 5 | China | UPLC-MS/MS | <LOQ–61.5 | 4.8 | 0.25 ng/mL | 0.75 ng/mL | >90% | [171] |
| ZEN | Fish feed | 11 | 11 | Europe | HPLC | 3–511 | 67.9 | 2.0 | 6.0 | 79% | [172] |
| ZEN | Pig feed | 17 | 1 | Korea | HPLC-MS/MS | | 124.8 (mg/kg) | | 3.1 | 106% | [173] |
| ZAL | Pig feed | 17 | 2 | Korea | HPLC-MS/MS | 4.7–6.7 (mg/kg) | | 0.6 | 2 | 95% | [173] |
| β-ZAL | Pig feed | 17 | 1 | Korea | HPLC-MS/MS | | 3.1 (mg/kg) | 0.3 | 1 | 104% | [173] |
| α-ZAL | Pig feed | 17 | 2 | Korea | HPLC-MS/MS | 2.3–2.5 (mg/kg) | | 0.4 | 1.3 | 98% | [173] |
| α-ZAL | Chicken feed | 13 | 2 | Korea | HPLC-MS/MS | 13.7–19.1 (mg/kg) | | 0.4 | 1.3 | 98% | [173] |
| β-ZAL | Cattle feed | 14 | 1 | Korea | HPLC-MS/MS | | 2.5 | 0.3 | 1 | 104% | [173] |
| ZEN | Poultry feed | 9 | 1 | Spane | UPLC-MS/MS | | | 25 | 50 | >100% | [174] |
| ZEN | Cattle feed | 6 | 1 | Spain | UPLC-MS/MS | | 52.2 | 25 | 50 | >100% | [174] |
| ZEN | Sheep feed | 17 | 3 | Spain | UPLC-MS/MS | 104.4–54.4 | | 25 | 50 | >100% | [174] |
| ZEN | Swine feed | 20 | 2 | Spain | UPLC-MS/MS | | | 25 | 50 | >100% | [174] |
| ZEN | Sow feed | 15 | 15 | Hungary | ELISA | 18–35 | 21 | 17 | 51 | >85% | [175] |
| ZEN | Boar feed | 15 | 15 | Hungary | ELISA | 19–192 | 71 | 17 | 51 | >85% | [175] |
| ZEN | Piglet feed | 15 | 15 | Hungary | ELISA | 18–40 | 24.4 | 17 | 51 | >85% | [175] |
| ZEN | Broiler feed | 100 | 63 | Thailand | LC-MS/MS | 2.2–263.5 | 84.3 | 0.78 | 2 | >93% | [176] |
| ZEN | Feed | 466 | 386 | Poland | HPLC-MS/MS | 0.07–1113 | 18.6 | 0.07 | 0.2 | | [177] |
| ZEN | Pig feed (powder) | 25 | 24 | China | HPLC | 10–835.4 | 272.1 | 1.5 | 10 | | [178] |
| ZEN | Pig feed (pellet) | 90 | 73 | China | HPLC | 10–3346 | 634 | 1.5 | 10 | | [178] |
| ZEN | Duck feed | 6 | 6 | China | HPLC | 10–2613.7 | 1718.3 | 1.5 | 10 | | [178] |
| ZEN | Maize | 30 | 13 | Poland | HPLC | n.d.–59.9 | 18.4 | 1 | | | [179] |
| ZEN | Pig feed (pellet) | 132 | 132 | China | HPLC | 10–4279.3 | 973.6 | 10 | 24 | | [180] |
| ZEN | Pig feed (powder) | 427 | 425 | China | HPLC | 10–10,437.6 | 947.2 | 10 | 24 | | [180] |
| ZEN | Soybean meal | 31 | 29 | China | HPLC | 10–6.9 | 4.2 | 10 | 24 | | [180] |
| ZEN | Corn bran | 8 | 8 | China | HPLC | 10–13.5 | 7.3 | 10 | 24 | | [180] |
| ZEN | Lactation sow feed | 13 | 13 | China | HPLC | 7.4–231 | 76 | 1.5 | 4 | 85% | [181] |
| ZEN | Gestating sow feed | 10 | 10 | China | HPLC | 9.2–149 | 63 | 1.5 | 4 | 85% | [181] |
| ZEN | Grower feed | 18 | 18 | China | HPLC | 7.1–150 | 59 | 1.5 | 4 | 85% | [181] |
| ZEN | Soybean meal | 11 | 6 | China | HPLC | LOQ–35.4 | 9 | 1.5 | 4 | 85% | [181] |
| ZEN | Feed, maize | 1113 | 884 | World | LC-MS/MS | >1–11,192 | | >1 | | | [182] |

**Table 1.** *Cont.*

| MYT/ISF | Food Commodity | Samples Tested | Positive Samples | Country | Detection Technique | Concentration Range (µg/kg) | Average (µg/kg) | LOD (µg/kg) | LOQ (µg/kg) | Mean Recovery | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ZEN | Poultry feed | 20 | 17 | Korea | HPLC | 5.2–147.5 | 35 | 1.3 | 8 | >75% | [183] |
| ZEN | Dairy cattle feed | 40 | 24 | South Africa | LC-QTOF-MS7MS | LOQ–28 | 2.8 | 0.04 | 0.12 | >150% | [184] |
| α-ZEL | Dairy cattle feed | 40 | 40 | South Africa | LC-QTOF-MS7MS | 1–13.2 | 4.8 | 0.19 | 0.63 | >99% | [184] |
| β-ZEL | Dairy cattle feed | 40 | 40 | South Africa | LC-QTOF-MS7MS | 0.7–4.7 | 2.4 | 0.19 | 0.64 | >99% | [184] |
| ZEN | Complete pig feed | 30 | 30 | Norway | HPLC | <3–217 | 37 | | | | [185] |
| α-ZEL | Animal feed | 77 | 5 | Egypt | LC-MS/MS | LOQ–8 | | 1.3 | 4.5 | 84% | [186] |
| β-ZEL | Animal feed | 77 | 28 | Egypt | LC-MS/MS | LOQ–60 | | 1.2 | 3.5 | 87% | [186] |
| ZEN | Animal feed | 77 | 71 | Egypt | LC-MS/MS | LOQ–791 | | 0.6 | 2.1 | 86% | [186] |
| ZEN | Finished feed | 146 | 94 | Africa | LC-MS/MS | LOQ–518 | | | | | [187] |
| ZEN | Finished feed | 301 | 173 | South Africa | LC-MS/MS | LOQ–386 | | | | | [187] |
| ZEN | Soy | 30 | 24 | Brazil | HPLC | LOQ–104 | 16.7 | 2 | 6 | >99% | [188] |
| ZEN | Soybean meal | 14 | 10 | Pakistan | HPLC | 0.15–120.9 | 18.9 | 0.05 | 0.15 | >85% | [189] |
| ZEN | Poultry feed | 11 | 9 | Pakistan | HPLC | 0.15–125.2 | 15.8 | 0.05 | 0.15 | >85% | [189] |
| ZEN | Poultry feed | 13 | 10 | Pakistan | HPLC | 0.15–118.4 | 19.6 | 0.05 | 0.15 | >85% | [189] |
| ZEN | Cattle feed | 174 | | Korea | HPLC-MS/MS | | 134.2 | 0.1–3 | 0.3–8 | >96% | [190] |
| ZEN | Swine feed | 160 | | Korea | HPLC-MS/MS | | 31.7 | 0.1–3 | 0.3–8 | >96% | [190] |
| ZEN | Poultry feed | 160 | | Korea | HPLC-MS/MS | | 37.9 | 0.1–3 | 0.3–8 | >96% | [190] |
| ZEN | Cow mixed feed | 34 | 28 | Thailand | ESI-MS/MS | 0.96–12.4 | 5.2 | 0.19 | | 60% | [40] |
| ZEN | Cow concentrate | 33 | 33 | Thailand | ESI-MS/MS | 2.5–55.6 | 24.3 | 0.19 | | 60% | [40] |
| ZEN | Feed | 61,413 | 27,559 | Global | | LOQ–105,000 | 55 * | | | | [167] |
| ZEN | Finished feed | 19,171 | 10,676 | Global | | LOQ–9432 | 41 * | | | | [167] |
| ZEN | Maize | 15,860 | 7992 | Global | | LOQ–16,495 | 77 * | | | | [167] |
| ZEN | Maize silage | 3735 | 1508 | Global | | LOQ–6239 | 84 * | | | | [167] |
| ZEN | Soybean grains | 1024 | 364 | Global | | LOQ–4336 | 43 * | | | | [167] |
| ZEN | Soybean meal | 1767 | 1072 | Global | | LOQ–3720 | 47 * | | | | [167] |
| ZEN | Wheat | 4925 | 1624 | Global | | LOQ–23,278 | 34 * | | | | [167] |
| ZEN | Barley | 3129 | 637 | Global | | LOQ–8952 | 25 * | | | | [167] |
| ZEN | Rice | 220 | 74 | Global | | LOQ–1530 | 60 * | | | | [167] |

**Table 1.** *Cont.*

| MYT/ISF | Food Commodity | Samples Tested | Positive Samples | Country | Detection Technique | Concentration Range (µg/kg) | Average (µg/kg) | LOD (µg/kg) | LOQ (µg/kg) | Mean Recovery | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DAI | Cow mixed feed | 34 | 17 | Thailand | ESI-MS/MS | 1013–3759 | 2024 | 0.5 | | 70% | [40] |
| Daidzin | Cow mixed feed | 34 | 16 | Thailand | ESI-MS/MS | 30–15,030 | 5025 | 0.8 | | 47% | [40] |
| GEN | Cow mixed feed | 34 | 18 | Thailand | ESI-MS/MS | 790–4255 | 2447 | 0.8 | | 85% | [40] |
| Genistin | Cow mixed feed | 34 | 16 | Thailand | ESI-MS/MS | 104–20,106 | 6824 | 0.8 | | 50% | [40] |
| GLY | Cow mixed feed | 34 | 18 | Thailand | ESI-MS/MS | 129–1474 | 652 | 0.8 | | 61% | [40] |
| Glycitin | Cow mixed feed | 34 | 15 | Thailand | ESI-MS/MS | 190–3113 | 1406 | 0.8 | | 56% | [40] |
| DAI | Cow concentrate | 33 | 21 | Thailand | ESI-MS/MS | 1344–4720 | 2678 | 0.5 | | 74% | [40] |
| Daidzin | Cow concentrate | 33 | 21 | Thailand | ESI-MS/MS | 16,520–42,736 | 24,157 | 0.8 | | 100% | [40] |
| GEN | Cow concentrate | 33 | 21 | Thailand | ESI-MS/MS | 1319–4927 | 3056 | 0.8 | | 107% | [40] |
| Genistin | Cow concentrate | 33 | 21 | Thailand | ESI-MS/MS | 21,000–57,912 | 33,637 | 0.8 | | 100% | [40] |
| GLY | Cow concentrate | 33 | 21 | Thailand | ESI-MS/MS | 495–1686 | 1033 | 0.8 | | 64% | [40] |
| Glycitin | Cow concentrate | 33 | 21 | Thailand | ESI-MS/MS | 4539–13,648 | 8489 | 0.8 | | 100% | [40] |
| Formononetin | Silage (clover) | 3 | 3 | Belgium | LC-MS/MS | 3.1–11.3 | | 0.16 ng/mL | 0.53 ng/mL | >79% | [191] |
| Biochanin A | Silage (clover) | 3 | 3 | Belgium | LC-MS/MS | 19–24.7 | | 0.15 ng/mL | 0.50 ng/mL | >47% | [191] |
| GEN | Silage (clover) | 3 | 2 | Belgium | LC-MS/MS | 3.9–6.2 | | 1.3 ng/mL | 4.4 ng/mL | >92% | [191] |
| DAI | Silage (clover) | 3 | 2 | Belgium | LC-MS/MS | 4.3–8.1 | | 0.15 ng/mL | 0.5 ng/mL | >80% | [191] |
| Formononetin | Silage (grass) | 3 | 3 | Belgium | LC-MS/MS | 14.3–49.1 | | 0.16 ng/mL | 0.53 ng/mL | >79% | [191] |
| Biochanin A | Silage (grass) | 3 | 2 | Belgium | LC-MS/MS | 6.8–24.1 | | 0.15 ng/mL | 0.50 ng/mL | >47% | [191] |
| GEN | Silage (grass) | 3 | 3 | Belgium | LC-MS/MS | 3.1–5.7 | | 1.3 ng/mL | 4.4 ng/mL | >92% | [191] |
| DAI | Silage (grass) | 3 | 2 | Belgium | LC-MS/MS | 2.8–10.8 | | 0.15 ng/mL | 0.5 ng/mL | >80% | [191] |
| Formononetin | Silage (cocksfoot) | 8 | 8 | Belgium | LC-MS/MS | 444.8–687.6 | | 0.16 ng/mL | 0.53 ng/mL | >79% | [191] |
| Biochanin A | Silage (cocksfoot) | 8 | 8 | Belgium | LC-MS/MS | 436.5–548.8 | | 0.15 ng/mL | 0.50 ng/mL | >47% | [191] |
| GEN | Silage (cocksfoot) | 8 | 8 | Belgium | LC-MS/MS | 105.8–256.6 | | 1.3 ng/mL | 4.4 ng/mL | >92% | [191] |
| DAI | Silage (cocksfoot) | 8 | 8 | Belgium | LC-MS/MS | 175.7–397.4 | | 0.15 ng/mL | 0.5 ng/mL | >80% | [191] |
| Daidzin | Soybean meal | 6 | | Argentina | HPLC | 494–785 | 596 | | | | [72] |
| DAI | Soybean meal | 6 | | Argentina | HPLC | 146–203 | 172 | | | | [72] |
| Genistin | Soybean meal | 6 | | Argentina | HPLC | 930–1274 | 1066 | | | | [72] |
| GEN | Soybean meal | 6 | | Argentina | HPLC | 69–110 | 82 | | | | [72] |
| Glycitin | Soybean meal | 6 | | Argentina | HPLC | 168–208 | 181 | | | | [72] |
| GLY | Soybean meal | 6 | | Argentina | HPLC | 152–279 | 190 | | | | [72] |

**Table 1.** *Cont.*

| MYT/ISF | Food Commodity | Samples Tested | Positive Samples | Country | Detection Technique | Concentration Range (µg/kg) | Average (µg/kg) | LOD (µg/kg) | LOQ (µg/kg) | Mean Recovery | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Daidzin | Soybean meal | 6 | | Brazil | HPLC | 248–403 | 298 | | | | [72] |
| DAI | Soybean meal | 6 | | Brazil | HPLC | 60–143 | 122 | | | | [72] |
| Genistin | Soybean meal | 6 | | Brazil | HPLC | 551–704 | 607 | | | | [72] |
| GEN | Soybean meal | 6 | | Brazil | HPLC | 26–100 | 81 | | | | [72] |
| Glycitin | Soybean meal | 6 | | Brazil | HPLC | 116–168 | 142 | | | | [72] |
| GLY | Soybean meal | 6 | | Brazil | HPLC | 53–93 | 67 | | | | [72] |
| Daidzin | Soybean meal | 6 | | USA | HPLC | 234–257 | 326 | | | | [72] |
| DAI | Soybean meal | 6 | | USA | HPLC | 25–76 | 53 | | | | [72] |
| Genistin | Soybean meal | 6 | | USA | HPLC | 410–688 | 535 | | | | [72] |
| GEN | Soybean meal | 6 | | USA | HPLC | 7–41 | 24 | | | | [72] |
| Glycitin | Soybean meal | 6 | | USA | HPLC | 73–137 | 113 | | | | [72] |
| GLY | Soybean meal | 6 | | USA | HPLC | 71–137 | 96 | | | | [72] |
| ZEN | Pastures | 18 | 9 | Austria | LC-MS/MS | 2.62–138 | 29.6 | | | | [170] |
| Biochanin A | Pastures | 18 | 16 | Austria | LC-MS/MS | 62.1–20,650 | 7060 | | | | [170] |
| DAI | Pastures | 18 | 15 | Austria | LC-MS/MS | 5.16–6110 | 926 | | | | [170] |
| Daidzin | Pastures | 18 | 6 | Austria | LC-MS/MS | 15.8–543 | 167 | | | | [170] |
| GEN | Pastures | 18 | 15 | Austria | LC-MS/MS | 28.4–17,550 | 2760 | | | | [170] |
| Genistin | Pastures | 18 | 9 | Austria | LC-MS/MS | 14.6–1630 | 311 | | | | [170] |
| GLY | Pastures | 18 | 15 | Austria | LC-MS/MS | 313–35,850 | 7470 | | | | [170] |

MYT = mycotoxin; ISF = isoflavone; LOD = limit of detection; LOQ = limit of quantification; ZEN = Zearalenone; α-ZAL = α-Zearalanol; β-ZAL = β-Zearalanol; α-ZEL = α-Zearalenol; β-ZEL = β-Zearalenol; GEN = Genistein; DAI = Daidzein; GLY = Glycitein; values marked with * are reported as median values of positive samples.

### *4.2. Spectrum 380® Method*

In most studies, occurrence data are limited to either myco- or phytoestrogens. In general, information on the co-occurrence of phytoestrogens is scare. BIOMIN has been conducting analyses to investigate the co-occurrence of feed contaminants including mycotoxins since 2004. Ten years later Spectrum 380® was launched in cooperation with the Institute of Bioanalytics and Agro-Metabolomics of the University of Natural Resources and Life Sciences, Vienna (BOKU). Spectrum 380® is a service to customers with the aim to monitor more than 700 mycotoxins and other secondary metabolites of bacteria and fungi, 300 pesticides and 150 veterinary drugs [192]. Since the inclusion of phytoestrogens in January 2019, 1694 feed samples were analyzed. These feed samples are either finished feed or feed components which will be mixed prior to use. Tables 2–5 provide descriptive statistics for the occurrence of ISF, ZEN, and ZEN metabolites in feed designated for different animal species.

**Table 2.** Occurrence of ISF and ZEN in aquaculture feed (Spectrum 380®). Please note that ZEN and ZEN-metabolite concentrations are provided in µg/kg whereas the ISF as feed constituents occur in higher concentrations and are reported in mg/kg (factor 1000 higher).

| | **Aqua Feed (*n* = 26)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **DAI** | **Daidzin** | **GEN** | **Genistin** | **GLY** | **Glycitin** | **ZEN *** |
| Positive samples (*n*) | 22 | 25 | 22 | 25 | 23 | 24 | 19 |
| Positive samples (%) | 85% | 96% | 85% | 96% | 88% | 92% | 73% |
| Mean (mg/kg) | 5.90 | 17.2 | 7.91 | 26.7 | 1.66 | 9.02 | 233 |
| Maximum (mg/kg) | 25.3 | 79.1 | 37.6 | 129 | 9.22 | 94.7 | 1045 |
| Third quartile (mg/kg) | 6.14 | 32.7 | 15.2 | 47.4 | 2.93 | 12.4 | 334 |
| Median (mg/kg) | 0.765 | 2.25 | 1.35 | 3.87 | 0.202 | 0.806 | 12.5 |
| First quartile (mg/kg) | 0.195 | 0.545 | 0.343 | 0.731 | 0.0298 | 0.335 | 3.82 |
| Concentration ratio ZEN/ISF | 0.04 | 0.01 | 0.03 | 0.01 | 0.14 | 0.03 | |
| Co-occurrence of ISF and ZEN (*n*) | 15 | 18 | 15 | 18 | 16 | 17 | |
| Co-occurrence of ISF and ZEN (% of samples) | 58 | 69 | 58 | 69 | 62 | 65 | |

Mycotoxins marked with * are reported in µg/kg; *n* = sample number; LOQ = limit of quantification; ISF = isoflavones; ZEN = Zearalenone; DAI = Daidzein; GEN = Genistein; GLY = Glycitein.

**Table 3.** Occurrence of ISF and ZEN and its metabolites in cattle feed (Spectrum 380®). Please note that ZEN and ZEN-metabolite concentrations are provided in µg/kg whereas the ISF as feed constituents occur in higher concentrations and are reported in mg/kg (factor 1000 higher).

| | **Cattle Feed (*n* = 542)** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Biochanin A** | **DAI** | **Daidzin** | **Formononetin** | **GEN** | **Genistin** | **GLY** | **Glycitin** | **α-ZEL *** | **β-ZEL *** | **ZEN *** |
| Positive samples (*n*) | 118 | 144 | 146 | 14 | 170 | 140 | 93 | 88 | 16 | 16 | 317 |
| Positive samples (%) | 22% | 27% | 27% | 3% | 31% | 26% | 17% | 16% | 3% | 3% | 58% |
| Mean (mg/kg) | 2.53 | 2.75 | 4.29 | 42.8 | 3.62 | 5.91 | 2.96 | 2.48 | 15.8 | 14.1 | 42.3 |
| Maximum (mg/kg) | 29.8 | 49.8 | 175 | 183 | 64.2 | 225 | 35.8 | 57.7 | 119 | 35.7 | 1305 |
| Third quartile (mg/kg) | 1.67 | 1.97 | 1.39 | 36.6 | 2.66 | 1.49 | 3.22 | 1.47 | 8.88 | 20.5 | 29.9 |
| Median (mg/kg) | 0.223 | 0.354 | 0.108 | 13.9 | 0.321 | 0.255 | 0.681 | 0.292 | 4.50 | 9.06 | 11.1 |
| First quartile (mg/kg) | 0.028 | 0.052 | 0.028 | 5.04 | 0.036 | 0.044 | 0.123 | 0.055 | 2.78 | 6.52 | 4.09 |
| Concentration ratio ZEN/ISF | 0.02 | 0.02 | 0.01 | 0.001 | 0.01 | 0.01 | 0.01 | 0.02 | | | |
| Co-occurrence of ISF and ZEN (*n*) | 60 | 85 | 86 | 8 | 100 | 78 | 57 | 58 | | | |
| Co-occurrence of ISF and ZEN (% of samples) | 11 | 16 | 16 | 1 | 18 | 14 | 11 | 11 | | | |

Mycotoxins marked with * are reported in µg/kg; LOQ = limit of quantification; Q1 = first quartile; Q3 = third quartile; ISF = isoflavone; ZEN = Zearalenone; α-ZEL = α-Zearalenol; β-ZEL = β-Zearalenol; DAI = Daidzein; GEN = Genistein; GLY = Glycitein.

**Table 4.** Occurrence of ISF and ZEN and its metabolites in pig feed (Spectrum 380®). Please note that ZEN and ZEN-metabolite concentrations are provided in µg/kg whereas the ISF as feed constituents occur in higher concentrations and are reported in mg/kg (factor 1000 higher).

| | **Pig Feed (*n* = 862)** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Biochanin A** | **DAI** | **Daidzin** | **Formononetin** | **GEN** | **Genistin** | **GLY** | **Glycitin** | **α-ZEL *** | **β-ZEL *** | **ZEN *** |
| Positive samples (*n*) | 78 | 334 | 362 | 16 | 345 | 351 | 303 | 316 | 11 | 14 | 390 |
| Positive samples (%) | 9% | 39% | 42% | 2% | 40% | 41% | 35% | 37% | 1% | 2% | 45% |
| Mean (mg/kg) | 4.18 | 5.98 | 40.3 | 1.66 | 9.15 | 63.3 | 2.89 | 18.5 | 25.0 | 10.7 | 115 |
| Maximum (mg/kg) | 316 | 61.9 | 358 | 15.7 | 140 | 442 | 163 | 118 | 105 | 36.0 | 9905 |
| Third quartile (mg/kg) | 0.018 | 8.01 | 60.0 | 0.109 | 11.4 | 86.9 | 2.34 | 21.6 | 34.8 | 13.8 | 40.0 |
| Median (mg/kg) | 0.008 | 3.17 | 34.0 | 0.056 | 4.69 | 49.9 | 0.795 | 12.7 | 7.84 | 6.99 | 13.6 |
| First quartile (mg/kg) | 0.003 | 1.05 | 2.36 | 0.031 | 1.23 | 6.91 | 0.369 | 4.10 | 4.87 | 5.52 | 4.96 |
| Concentration ratio ZEN/ISF | 0.027 | 0.019 | 0.003 | 0.069 | 0.013 | 0.002 | 0.040 | 0.006 | | | |
| Co-occurrence of ISF and ZEN (*n*) | 56 | 252 | 263 | 13 | 254 | 257 | 236 | 246 | | | |
| Co-occurrence of ISF and ZEN (% of samples) | 6 | 29 | 31 | 2 | 29 | 30 | 27 | 29 | | | |

Mycotoxins marked with * are reported in µg/kg; LOQ = limit of quantification; Q1 = first quartile; Q3 = third quartile; ISF = isoflavone; ZEN = Zearalenone; α-ZEL = α-Zearalenol; β-ZEL = β-Zearalenol; DAI = Daidzein; GEN = Genistein; GLY = Glycitein.

**Table 5.** Occurrence of ISF and ZEN and its metabolites in poultry feed (Spectrum 380®). Please note that ZEN and ZEN-metabolite concentrations are provided in µg/kg whereas the ISF as feed constituents occur in higher concentrations and are reported in mg/kg (factor 1000 higher).

| | **Poultry Feed (*n* = 263)** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Biochanin A** | **DAI** | **Daidzin** | **Formononetin** | **GEN** | **Genistin** | **GLY** | **Glycitin** | **α-ZEL *** | **β-ZEL *** | **ZEN *** |
| Positive samples (*n*) | 13 | 176 | 205 | 2 | 191 | 212 | 159 | 168 | 10 | 18 | 177 |
| Positive samples (%) | 5% | 67% | 78% | 1% | 73% | 81% | 60% | 64% | 4% | 7% | 67% |
| Mean (mg/kg) | 0.016 | 7.57 | 56.9 | 0.237 | 10.4 | 78.2 | 2.93 | 26.8 | 5.33 | 9.80 | 53.1 |
| Maximum (mg/kg) | 0.124 | 53.7 | 342 | 0.306 | 102 | 465 | 37.2 | 127 | 12.6 | 58.7 | 873 |
| Third quartile (mg/kg) | 0.011 | 10.2 | 86.4 | 0.271 | 14.6 | 131 | 3.32 | 37.9 | 7.45 | 6.13 | 50.4 |
| Median (mg/kg) | 0.007 | 6.46 | 39.3 | 0.237 | 8.71 | 60.4 | 1.86 | 19.4 | 4.24 | 2.74 | 17.7 |
| First quartile (mg/kg) | 0.004 | 1.98 | 0.790 | 0.202 | 1.12 | 0.453 | 0.67 | 6.49 | 1.75 | 1.66 | 6.95 |
| Concentration ratio ZEN/ISF | 3.2 | 0.007 | 0.001 | 0.2 | 0.005 | 0.001 | 0.018 | 0.002 | | | |
| Co-occurrence of ISF and ZEN (*n*) | 11 | 142 | 155 | 1 | 149 | 158 | 129 | 136 | | | |
| Co-occurrence of ISF and ZEN (% of samples) | 4 | 54 | 59 | 0 | 57 | 60 | 49 | 52 | | | |

Mycotoxins marked with * are reported in µg/kg; LOQ = limit of quantification; Q1 = first quartile; Q3 = third quartile; ISF = isoflavone; ZEN = Zearalenone; α-ZEL = α-Zearalenol; β-ZEL = β-Zearalenol; DAI = Daidzein; GEN = Genistein; GLY = Glycitein.

#### 4.2.1. Aquaculture Feed

Between January 2019 and April 2021, a total of 26 aquaculture feed samples from different countries were analyzed (Table 2) and 92% of those were classified as finished feed samples. ISF were detected in 96% of these samples. The individual ISF were present in 85%–96% of the feed samples (Table 2). Only GEN, DAI, and GLY and their glycosides were detected, with genistin and daidzin being the most prevalent (Table 2), suggesting that the major ISF source was soybean. Glucosides occurred in higher concentrations than their aglycons (Table 2). The mean and maximum concentrations were 7.91 and 37.6 mg/kg, respectively, for GEN, and 26.7 and 129 mg/kg, respectively, for its glucoside genistin. Slightly lower values were observed for DAI and its glucoside daidzin (Table 2).

In aqua feed, ZEN occurred in the medium to high µg/kg range with mean and maximum concentrations of 233 µg/kg and 1045 µg/kg, respectively. The prevalence of ZEN was high with detectable concentrations in 73% of the samples. The concentrations of phytoestrogens were at least one order of magnitude higher than those of ZEN (Table 2). For example, the ratio of medians was 0.04 for ZEN/DAI and 0.03 for ZEN/GEN. ISF and ZEN co-occurred in 58–69% of the samples.

#### 4.2.2. Cattle Feed

ISF were detected in 43% of cattle feed samples collected from January 2019 to April 2021 ($n$ = 542). The prevalence of the individual ISF in cattle feed ranged from 2.6% to 31% (Table 3). In addition to the ISF that were detected in the aquaculture feed samples (Section 4.2.1; Table 2), biochanin A and formononetin were detected. Formononetin even reached higher mean and median concentrations than the other ISF (42.8 mg/kg and 13.9 mg/kg, respectively; Table 3). Feed for cattle often consist of different grasses, as well as clover species rich in biochanin A and formononetin, which could explain the high concentrations of these ISF in our samples.

Detectable levels of ZEN were present in 58% of cattle feed samples with mean, median, and maximum concentrations of 42.3 µg/kg, 11.1 µg/kg and 1305.0 µg/kg, respectively. ZEN metabolites (α-ZEL) and β-zearalenol (β-ZEL) were detected in 3% of the tested samples. Median concentrations of phytoestrogens were at least one order of magnitude higher than those of ZEN and its metabolites α-ZEL and β-ZEL (Table 3). The ratios of the median concentration of ZEN to the median concentration of each individual ISF were $\leq$0.02 (Table 3). ISF and ZEN co-occurred in 24% of cattle feed samples (percentage of samples co-contaminated with ZEN and individual ISF: 1.5–18.5%). Therefore, co-occurrence of ZEN and ISF was less frequently detected than in aquaculture (Table 2), pig (Section 4.2.3) and poultry (Section 4.2.4) feed samples.

#### 4.2.3. Pig Feed

More than 40% of the samples analyzed in this study were specified as pig feed ($n$ = 862). ISF were detected in up to 45% of the pig feed samples, with daidzin and genistin as well as their aglycones being the most prevalent ones (Table 4). The detected mean, median, and maximum concentrations were 9.2, 4.7 and 140.0 mg/kg, respectively, for GEN and 63.3, 49.9 and 442.0 mg/kg, respectively, for its glucoside genistin; DAI and its glucoside daidzin were detected at similar but somewhat lower concentrations (Table 4). Biochanin A and formononetin were only present in 9% and 2% of samples, respectively, indicating that the major source of ISF in the pig feed samples analyzed in this study were soybeans.

ZEN occurred in the low to medium µg/kg range in most samples, with mean, median, and maximum concentrations of 115.0 µg/kg, 13.6 µg/kg and 9905.0 µg/kg, respectively (Table 4). The prevalence of ZEN was relatively high with detectable concentrations in 45% of the samples. ZEN metabolites α-ZEL and β-ZEL were detected in only 1.2% and 1.6% of the tested samples, respectively. ISF and ZEN co-occurred in 32% of the samples. Co-occurrence of ZEN with each individual ISF was detected in 1.5–30% of the samples (Table 4). Median ratios of ZEN/ISF did not exceed 0.07 (Table 4).

Pig feed was further divided into feed for 'pig' ($n$ = 463), 'pig-boar' ($n$ = 7), 'pig-finisher' ($n$ = 10), 'pig-grower' ($n$ = 33), 'pig-piglet' ($n$ = 175) and 'pig-sow' ($n$ = 172) (Tables S1–S6 are provided in the Supplementary Materials). The samples that were specified as 'pig' did not show a high incidence of either ISF (highest for daidzin 14%) or ZEN (16%) and its metabolites (Supplementary Table S1). However, the mean and median values for ISF were comparable with the values obtained for all pig feed samples (Table 4; Supplementary Tables S1–S6), whereas in the case of ZEN, the mean (318 µg/kg) exceeded the mean detected for all pig feed samples. The co-occurrence of ISF and ZEN for these samples was low, with the highest incidence of 6% for daidzin and ZEN. The situation was different for samples classified as 'pig-piglet', where ISF and ZEN were detected in up to 71% and 86% of samples, respectively. Mean, median and maximum concentrations were highest for genistin (64.6, 64.9 and 260 mg/kg), followed by daidzin and glycitin (Supplementary Table S5). The concentration of glycosides was 7–9 times higher compared to that of their aglycones, where the highest mean value was also observed for GEN, followed by DAI and GLY. The mean and maximum concentrations for ZEN were 43.1 µg/kg and 595 µg/kg. ZEN co-occurred with individual soy ISF in 60–62% of the samples (Supplementary Table S5). Soy ISF were even more prevalent in 'pig-sow' feed. Here, up to 80% of all samples contained soy ISF (Supplementary Table S1). Biochanin A und formononetin were present only in a small number of samples (Supplementary Table S6). Detectable levels of ZEN were present in 73% of the 'pig-sow' feed samples with mean, median and maximum concentrations of 100 µg/kg, 17.3 µg/kg and 3262 µg/kg, respectively.

The European Commission published guidance values for ZEN that should not be exceeded in certain feed commodities [36]. The mean values for the 'pig-piglet' and 'pig-sow' samples were below the guidance values for these two groups (100 and 250 µg/kg, respectively). Only the mean value of the feed classified as 'pig' exceeded the guidance values. Of all farm animals, young piglets are the most sensitive to ZEN regarding its effects on the reproduction [32]. As early as 1928, the occurrence of hyperestrogenism in swine, characterized by swelling and erythema of the vulva and uterine prolapse in gilts as well as atrophy of testicles and enlargement of nipples, could be associated with the consumption of ZEN contaminated cereals [193]. Although the feed samples declared as pig are not specified for any life stage, young piglets could be exposed to such high concentrations of ZEN, potentially adversely affecting their health.

#### 4.2.4. Poultry Feed

In total, 263 poultry feed samples were analyzed for the presence of ISF and ZEN and its metabolites (Table 5). Soy ISF were present in 88% of the feed samples (occurrence of individual ISF: 60%–81%; Table 5). Aglycones were again detected in lower concentrations by a factor of 7–10 compared to the respective glycosides (Table 5). The mean, median, and maximum concentrations were 10.4 mg/kg, 8.71 mg/kg and 102 mg/kg for GEN and 78.2 mg/kg, 60.4 mg/kg and 465 mg/kg for its glucoside genistin. The corresponding values for DAI and its glucoside daidzin were similar but somewhat lower compared to GEN and genistin (Table 5). Formononetin and biochanin A showed only low prevalence in poultry feed (0.8 and 5%, respectively). The prevalence of ZEN was high, with detectable concentrations in 67% of the samples and mean, median, and maximum concentrations of 53.1 µg/kg, 17.7 µg/kg and 873 µg/kg, respectively. Soy ISF and ZEN co-occurred in a high number of poultry samples (i.e., 63%; co-occurrence of individual ISF with ZEN: 49–60%). The ratio of median concentrations of ZEN/soy ISF were $\leq$0.007.

Similar to pig feed samples, poultry feed was further divided into subgroups: 'poultry' ($n$ = 63), 'poultry-breeder' ($n$ = 31), 'poultry-broiler' ($n$ = 124) and 'poultry-layer' ($n$ = 38) (the respective Tables S7–S11 are provided in the Supplementary Materials). For the different poultry feed subgroups, the distribution, mean and maximum values for both ISF and ZEN and its metabolite were similar when compared to all poultry feed samples (Supplementary Table S2).

## 5. Conclusions

ISF can cause both positive and negative effects in animals, depending on the species, the age and sex of the animals, and the dose and the frequency of exposure. Moderate concentrations of ISF, which occur naturally in feed, can have positive effects on growth performance, laying performance, and milk production. However, also negative effects on growth and laying performance have occasionally been observed. Furthermore, in ruminants, pigs, and fish, high concentrations of ISF were shown to negatively affect reproductive health.

Based on a literature review and analysis of 1694 feed samples with the Spectrum 380® method, it is safe to say that both the prevalence and the concentrations of ISF are high in animal feed. The predominant ISF vary between feed destined for different animal species. Although formononetin is the dominant ISF in cattle, GEN and DAI are the most abundant ISF in feed destined for other species. ISF frequently co-occur with ZEN in animal feed. Same as ISF, ZEN can have a negative effect on reproductive health. It has already been reported that combinations of even small amounts of ISF and ZEN lead to an increased estrogenic effect when compared to the potency of the single substances in vitro [41]. Such an increase in estrogenic potency resulting from combinations of these two substance classes should be investigated further in the future. Clarification is needed whether co-occurrence of these estrogen-active compounds might result in a critical shift of the endocrine activity.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/toxins13120836/s1, Table S1: Occurrence of ISF and ZEN in pig feed (Spectrum 380®), Table S2: Occurrence of ISF and ZEN in pig-boar feed (Spectrum 380®), Table S3: Occurrence of ISF and ZEN in pig-finisher feed (Spectrum 380®), Table S4: Occurrence of ISF and ZEN in pig-grower feed (Spectrum 380®), Table S5: Occurrence of ISF and ZEN in pig-piglet feed (Spectrum 380®), Table S6: Occurrence of ISF and ZEN in pig-sow feed (Spectrum 380®), Table S7: Occurrence of ISF and ZEN in poultry feed (Spectrum 380®), Table S8: Occurrence of ISF and ZEN in poultry-breeder feed (Spectrum 380®), Table S9: Occurrence of ISF and ZEN in poultry-broiler feed (Spectrum 380®), Table S10: Occurrence of ISF and ZEN in poultry-layer feed (Spectrum 380®), Table S11: Occurrence of ISF and ZEN in poultry-turkey feed (Spectrum 380®).

**Author Contributions:** Conceptualization, D.G., E.V., B.N. and D.M.; writing—original draft preparation, D.G.; writing—review and editing, E.V., B.N., A.M. and D.M.; visualization, D.G.; funding acquisition, E.V., B.N. and D.M. All authors read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Austrian Research Promotion Agency (FFG) and BIOMIN Holding GmbH through the Bridge project "ISOMYCOTOX – Combinatory endocrine activity of mycoestrogens and soy isoflavones in porcine feed" (project number 880656). The APC was funded by BIOMIN Holding GmbH.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** We want to acknowledge Michael Sulyok for providing us with the Spectrum 380® data. Furthermore, we want to thank Christiane Gruber-Dorninger for her input and suggestions for this review.

**Conflicts of Interest:** The authors declare no conflict of interest.

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8.2 Publication 2:
“Estrogenic *in vitro* evaluation of zearalenone and its phase I and II metabolites in combination with soy isoflavones”

Archives of Toxicology (2022) 96:3385–3402
https://doi.org/10.1007/s00204-022-03358-3

BIOLOGICS

# Estrogenic in vitro evaluation of zearalenone and its phase I and II metabolites in combination with soy isoflavones

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Received: 24 May 2022 / Accepted: 3 August 2022 / Published online: 20 August 2022


## Abstract

Humans and animals are exposed to multiple substances in their food and feed that might have a negative health impact. Among these substances, the *Fusarium* mycoestrogen zearalenone (ZEN) and its metabolites α-zearalenol (α-ZEL) and α-zearalanol (α-ZAL) are known to possess endocrine disruptive properties. In a mixed diet or especially animal feed, these potential contaminants might be ingested together with naturally occurring phytoestrogens such as soy isoflavones. So far, risk assessment of potential endocrine disruptors is usually based on adverse effects of single compounds whereas studies investigating combinatorial effects are scarce. In the present study, we investigated the estrogenic potential of mycoestrogens and the isoflavones genistein (GEN), daidzein (DAI) and glycitein (GLY) as well as equol (EQ), the gut microbial metabolite of DAI, in vitro alone or in combination, using the alkaline phosphatase (ALP) assay in Ishikawa cells. In the case of mycoestrogens, the tested concentration range included 0.001 to 10 nM with multiplication steps of 10 in between, while for the isoflavones 1000 times higher concentrations were investigated. For the individual substances the following order of estrogenicity was obtained: α-ZEL > α-ZAL > ZEN > GEN > EQ > DAI > GLY. Most combinations of isoflavones with mycoestrogens enhanced the estrogenic response in the investigated concentrations. Especially lower concentrations of ZEN, α-ZEL and α-ZAL (0.001—0.01 nM) in combination with low concentrations of GEN, DAI and EQ (0.001—0.1 µM) strongly increased the estrogenic response compared to the single substances.

**Keywords** Isoflavones · Zearalenone · Phytoestrogens · Mycoestrogens · Combinatory toxicology · Endocrine disruptors

## Introduction

In the last decade, dietary habits have changed in western countries. According to an Australian survey plant-based diets gained in popularity and the number of vegetarians or vegans in the population increased (Roy Morgan 2016). For instance, the percentage of vegetarians and vegans in Germany doubled from 5 to 10% between 2020 and 2021. As a consequence, the consumption of alternatives to animal products increased (BMEL 2021). Many of these plant alternatives are based on soy products as the primary substitute for meat due to the high protein content and potential health benefits which are associated with the presence of isoflavones (ISF) like genistein (GEN), glycitein (GLY), daidzein (DAI) and its gut microbial metabolite equol (EQ) (Fig. 1) (Qin et al. 2022). Epidemiological studies in humans suggest that a high intake of ISF may be associated with several positive health effects. For example, in Asian countries where soy is part of a traditional diet, the risk of developing breast, prostate and colorectal cancers is lower compared to low soy-consuming countries (Wu et al. 2008; Yan and Spitznagel 2009; Yan et al. 2010). Other studies claim various clinical benefits of ISF intake including a reduction in the incidental onset of certain types of diseases such as coronary heart disease, osteoporosis and memory loss (Tham et al. 1998; Sarkar and Li 2003; Sacks et al. 2006; Zheng et al. 2016). However, in vitro studies indicate proliferative effects of ISF on estrogen-sensitive cancer cells (Matsumura

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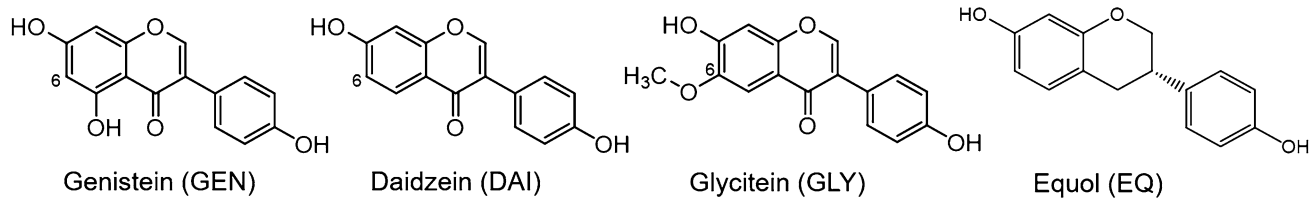


**Fig. 1** Chemical structures of glycitein (GLY), genistein (GEN), daidzein (DAI) and S-equol (EQ) (created with chem-space.com)

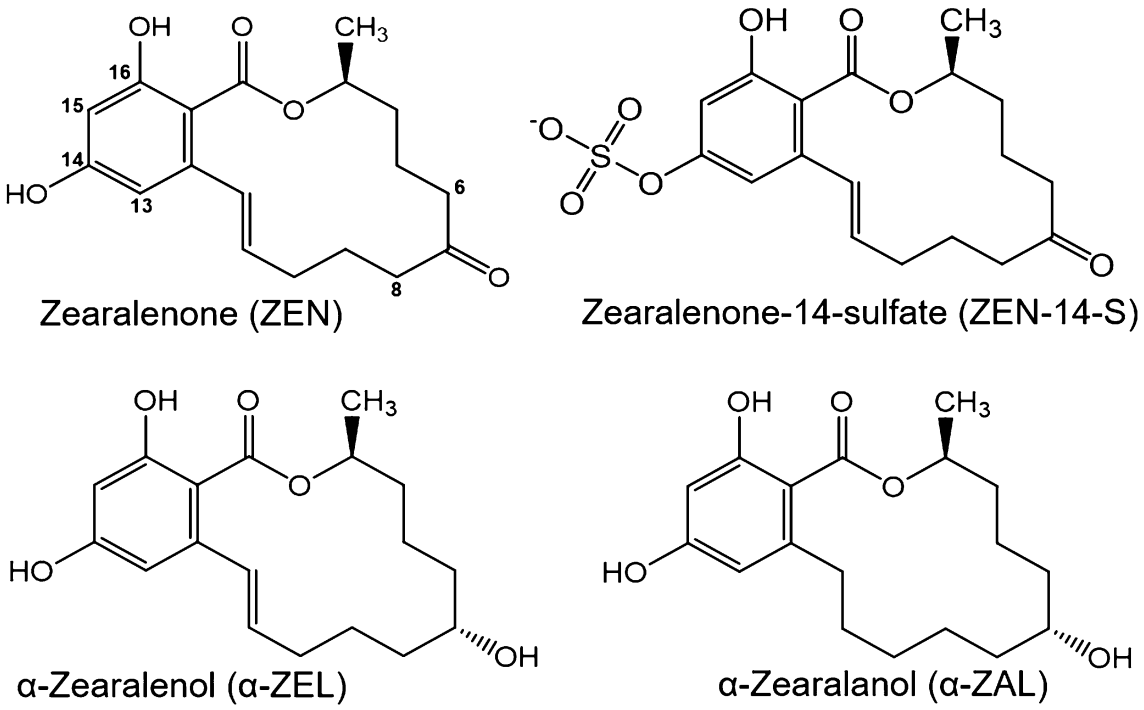


**Fig. 2** Chemical structures of zearalenone (ZEN), zearalenone-14-sulfate (ZEN-14-S), α-zearalenol (α-ZEL) and α-zearalanol (α-ZAL) (created with chem-space.com)

et al. 2005). Although the health effects are still controversially discussed, ISF or ISF-containing preparations are used as hormone replacement therapy in postmenopausal women (Carusi 2000; Aguiar and Barbosa 2014).

Regarding animal feed, soy is used for decades as the main protein source due to its high protein content (Goldsmith 2008) and several beneficial properties associated with ISF, such as growth promoting, improved antioxidative and immune functions (Grgic et al. 2021). However, there are also reports which indicate that a high intake of ISF might lead to negative health effects, mainly affecting the reproductive tract of female farm animals (Bennetts et al. 1946; Hashem and Soltan 2016), e.g. infertility, uterine prolapse and swelling of mammary glands and vulva.

ISF were described to co-occur frequently together with the mycotoxin zearalenone (ZEN) and several of its metabolites (Fig. 2) (up to 60%) in various animal feeds (Grgic et al. 2021; Penagos-Tabares et al. 2021). ZEN is a secondary fungal metabolite produced by *Fusarium* species and is commonly found in grains and legumes (Grgic et al. 2021). ZEN is known to have multiple toxic effects on humans and animals (Kuiper-Goodman et al. 1987; Fink-Gremmels and Malekinejad 2007; Kowalska et al. 2016; Rogowska et al. 2019). However, its most prominent effect is its ability to act as an endocrine disruptor. Its reduced phase I metabolite α-zearalenol (α-ZEL) is known to have even stronger estrogenic effects, whereas its phase II metabolites like the plant metabolite ZEN-14-glucoside are described to have lost their estrogenic properties (Molina-Molina et al. 2014; Dellafiora et al. 2017).

Both, ISF and ZEN represent xenoestrogens based on the structural and functional similarity to the endogenous hormone 17-β-estradiol (E2) (Fig. 3), which eventually bind and activate the estrogen receptors (ER), but with different

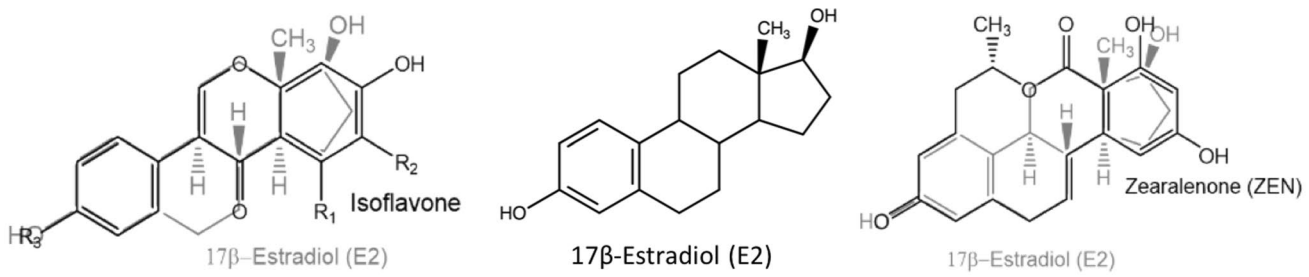


**Fig. 3** Chemical structure of zearalenone (ZEN) and isoflavone (ISF) scaffold in comparison, with 17-β-estradiol (E2) (created with chem-space.com)

affinity to the different ER isoforms α and β. ZEN is known to favorably bind to the ERα, whereas ISF have a higher affinity to the ERβ (Kuiper et al. 1998). The different binding affinity properties to the ERs of these two classes of substances might cause an enhanced estrogenic effect when they occur together (Vejdovszky et al. 2017b).

According to the scientific opinion of the European Food Safety Authority (EFSA) from 2011, ZEN exposure is quite high for certain populations and these values might be even higher for vegetarians and vegans (EFSA 2011). Therefore, regulatory limits for ZEN in different food and feed stuff have been established. For instance, in maize intended for direct human consumption and for feed designed for piglets this value should not exceed 100 µg/kg (EFSA 2011; European Commission 2016, 2022). However, risk assessments are still predominantly based on the toxicological data of single substances, which often underestimate the toxicological potential of certain mixtures. Data on combinatorial effects are still scarce and therefore, to ensure extensive health protection and food safety, further studies are required. Our aim was to provide a more detailed insight into the estrogenic properties of combinations between the mycoestrogen ZEN and its metabolites, with the phytoestrogens GEN, GLY, DAI and EQ.

## Material and methods

### Materials

Cell culture 96-well plates and flasks were obtained from Sarstedt (Nürnbrecht, Germany). Cell culture media (Minimal Essential Medium (MEM) and Dulbecco's Modified Eagle Medium/F12 (DMEM/F-12) without phenol red) and supplements (fetal bovine serum (FBS), charcoal-stripped FBS (CD-FBS), L-glutamine and penicillin–streptomycin (P/S)) were purchased from Gibco, Thermo Fisher Scientific, (Waltham/MA, USA). Zearalenone (ZEN), α-zearalenol (α-ZEL), α-zearalanol (α-ZAL), 17-β-estradiol (E2), 4-nitrophenylphosphate, diethanolamine, magnesium chloride and sulforhodamine B (SRB) were obtained from Sigma Aldrich Chemie GmbH (Schnelldorf, Germany), whereas daidzein (DAI), equol (EQ), genistein (GEN) and glycitein (GLY) were purchased from Extrasynthese (Genay Cedex, France). Dimethly sulfoxide (DMSO), NaCl, KCl, $Na_2HPO_4$, $Na_2HPO_4 \times 2\ H_2O$ and $KH_2PO_4$ were purchased from Roth (Karlsruhe, Germany). ZEN-4-sulfate ammonium salt was obtained from Santa Cruz Biotechnology (Dallas/TX, USA) and is the same compound as ZEN-14-sulfate (ZEN-14-S) using the newer International Union of Pure and Applied Chemistry (IUPAC) numbering system (Metzler 2011). The CellTiter-Blue® Cell Viability Assay Kit was purchased from Promega Corporation (Madison/WI, USA).

### Cell line

The human endometrial adenocarcinoma cell line "Ishikawa" was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Wiltshire, United Kingdom). Cell stocks were stored in liquid nitrogen containers and two weeks prior to the start of the in vitro experiments, cells were taken in culture. They were cultivated in an incubator at 37 °C with 5% $CO_2$ and 95% humidity using the growth medium consisting of MEM, supplemented with 5% (*v/v*) heat-inactivated FBS, 1% L-glutamine and 1% P/S. Cells of passage number 5 were split at a confluency of about 80% and kept in culture up to the maximum passage number of 40. Before the assays were performed, the growth medium was removed and replaced with the assay medium consisting of DMEM/F-12 supplemented with 5% CD-FBS and 1% P/S.

### Alkaline phosphatase assay

The assays were performed in 96-well plates and per well, 15 000 or 10 000 Ishikawa cells in the assay medium were seeded and grown for 24 or 48 h, respectively. Incubation was carried out for 48 h with different concentrations of ZEN, α-ZEL, α-ZAL, ZEN-14-S, GEN, DAI, EQ and GLY as single substances or in the respective combinations at

different concentrations. The substances were solved in DMSO in 200 times higher concentrations than the tested concentration, followed by dilution in the assay medium. In the case of single substance testing and for solvent control, DMSO was added to reach 1% in the final incubation solutions. The concentration ranges were 0.001 to 10 nM in the case of ZEN-metabolites and 0.001 to 10 µM in the case of ISF, with 1:10 dilution steps in between. An exception was GLY where the following concentrations were applied 0.01, 0.1, 1, 10 and 20 µM. E2 (1 nM) served as a positive control. All experiments were performed at least in five independent biological replicates (measurements with different cell passages) with technical triplicates (repeated measurements with the same cell passage) each. Since each ISF was measured with every mycoestrogen and the single substances were included on all plates of the respective combinations, at least 20 biological replicates of the respective single substance in technical triplicates were obtained.

Following the 48 h incubation, the supernatants were discarded and all wells were washed three times with 150 µL phosphate-buffered saline (PBS) per well. After the PBS was completely removed, the plate was placed in the freezer at −80 °C for at least 20 min. During this procedure, the cells were lysed, and the alkaline phosphatase (ALP) was released. The plate was thawed for five minutes at room temperature before 50 µL of the ALP buffer (containing 5 mM 4-nitrophenylphosphate, 1 M diethanolamine and 0.24 mM $MgCl_2$) was added in the dark. After five minutes at room temperature, the plate was placed in the plate reader and the absorbance was measured at 405 nm every 2 min for 1 h at 37 °C using a Victor V3 1240 Multilabel Counter from Perkin Elmer (Waltham/MA, USA) or a Cytation 3 Cell Imaging Multi-Mode Reader from Biotek® (Winooski/VM, USA). The activity of the ALP was calculated as the slope of the curve, obtained by the measurements monitored over 1 h. Final results were referred to the solvent and positive control which were set to 0 and 100%, respectively.

## Coupled CellTiter-Blue® and SRB cytotoxicity assay

After cell seeding (15 000 or 10 000 cells/well) and growing for 24 h or 48 h, respectively, the cells were incubated with the incubation solutions. These were prepared as described for the ALP assay and the same single substances and combinations thereof were incubated for 48 h. As solvent control 1% DMSO was used. All experiments were performed at least in five independent biological replicates with technical triplicates each, resulting in at least 20 biological replicates for the single substances. After the incubation time (48 h) the following steps were carried out in the dark. The medium was aspirated and 100 µL of a CellTiter-Blue® (CTB) incubation solution (1:10 dilution of CTB reagent and DMEM/F-12 (5% CD-FBS, 1% P/S)) was added. After an additional incubation time of 50 min with the CTB solution, 90 µL of each well were transferred to a new, black 96-well plate. The black plate was directly measured by an excitation wavelength of 560 nm and emission of 590 nm with a gain of 65 using the Victor V3 1240 Multilabel Counter from Perkin Elmer (Waltham/MA, USA) or the Cytation 3 Cell Imaging Multi-Mode Reader from Biotek® (Winooski/VT, USA). Final results were referred to as the solvent control in percentage.

The remaining 10 µL of the initial 96-well plate were aspirated, and cells were fixed with 10 µL of a 50% (*w/v*) trichloroacetic acid solution in distilled water for one hour at 4 °C. Thereafter, the 96-well plate was washed four times with tap water and dried overnight in the dark. Afterwards, 50 µL of the SRB solution (4 g SRB reagent solved in 1 L distilled water containing 1% acetic acid) were added to each well. After one-hour staining in the dark at room temperature, the coloring solution was discarded, and the plate was washed twice with tap water and twice with 1% acetic acid. After another drying step overnight in the dark, the dye was dissolved under alkaline conditions in 100 µL Tris base (0.30 g tris(hydroxymethyl-) aminomethane solved in 250 mL distilled water) by shaking for 5 min in the plate reader (Victor V3 1240 Multilabel Counter from Perkin Elmer (Waltham/MA, USA) or the Cytation 3 Cell Imaging Multi-Mode Reader from Biotek® (Winooski/VT Vermont, USA)). Subsequently, the absorbance was measured at 570 nm. As for the CTB, the final results were referred to the solvent control in percentage.

## Statistics

Measurements of single substances and their combinations for the ALP and the cytotoxicity assays were conducted each in technical triplicates and in at least five independent biological replicates ($n \geq 5$).

Biological replicates were tested for outliers according to Nalimov and for normality with the Kolmogorov–Smirnov test. Identified outliers were excluded in the calculation of the mean values and the standard deviations. At maximum, three outliers were excluded for the single substances resulting in at least 17 biological replicates and a maximum of one outlier was excluded for the combinations resulting in at least 4 biological replicates.

Statistical analyses and plotting of data were performed with the software Origin Pro® 2021, with significance levels of 5%, 1% and 0.1%, respectively (#, $x = p < 0.05$; ##, $xx = p < 0.01$; ###, $xxx = p < 0.001$). Significant differences were evaluated via one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test. Cytotoxicity results were evaluated by using one-way and two-way Student's *t*-test.

### Combination Index (CI)

To assess possible interactions between multiple compounds, like synergistic, antagonistic or additive effects, the mathematical combination index (CI) method developed by Chou and Talalay (Chou and Talalay 1981) was used. The CI is based on a variety of statistical equations including the mass-action law, the Michaelis–Menten equation (for enzyme kinetics), the Henderson-Hasselbalch equation (for pH ionization), the Hill equation (for ligand binding saturation) and the Scatchard equation (for receptor binding). The basis of the calculation of the CI is the median effect equation (MEE) (see Formula 1), which describes the relationship between dose and effect (Chou 2006).

$$\frac{f_a}{f_u}=\left(\frac{D}{D_m}\right)^m \quad (1)$$

$f_a$: affected fraction by the dose.
$f_u$: unaffected fraction = 1–$f_a$.
$D$: dose.
$D_m$: dose at mean effect (e.g., half-maximal effect concentration ($EC_{50}$)).
$m$: coefficient standing for the shape of the relationship between dose and effect, whereby $m = 1$ indicates hyperbolic, $m > 1$ sigmoidal and $m < 1$ flat sigmoidal dose-effect curves.

To calculate the CI, the MEE is transformed into the following equation (Chou 2006):

$$\mathrm{CI}=\frac{(D)_1}{(D_x)_1}+\frac{(D)_2}{(D_x)_2}=\frac{(D)_1}{(D_m)_1\left[\frac{f_a}{1-f_a}\right]^{\frac{1}{m}}}+\frac{(D)_2}{(D_m)_2\left[\frac{f_a}{1-f_a}\right]^{\frac{1}{m}}} \quad (2)$$

$f_a$: affected fraction by the dose.
$D$: dose.
$(D_x)_1$: dose of single substance 1 that has the same effect as the combination of $(D)_1+(D)_2$.
$(D_x)_2$: dose of single substance 2 that has the same effect as the combination of $(D)_1+(D)_2$
$D_m$: dose at mean effect (e.g., $ED_{50}$).
$m$: coefficient standing for the shape of the relationship between dose and effect, whereby $m = 1$ indicates hyperbolic, $m > 1$ sigmoidal and $m < 1$ flat sigmoidal dose-effect curves.

By calculating the CI, a statement about the effect induced by several compounds can be made. A CI value of 1 indicates an additive effect, whereas CI < 1 indicates synergism and CI > 1 antagonism (Chou 2006). The more detailed subdivision of synergism and antagonism according to Chou is illustrated in Table 1 (Chou 2006).

For applying the CI some requirements have to be fulfilled. Besides a monotonic dose–response curve, constant ratios of combinations should be used to receive the most exact evaluation of the CI. Furthermore, it is important that the maximum effects are the same for all combinations (Chou 2006; Vejdovszky et al. 2017b). Therefore, the highest ALP concentration of 115%, achieved by the combination of 0.01 nM ZEN with 1 µM EQ was set to 1. Since the CI analysis is only feasible with relative values between 0 and 1, all effects were relativized to the lowest and highest value of −26% and 115%, respectively.

**Table 1** Description of synergism and antagonism in combination studies with the combination index (CI)

| Range of CI | Description |
|---|---|
| < 0.10 | Very strong synergism |
| 0.10–0.30 | Strong synergism |
| 0.30–0.70 | Synergism |
| 0.70–0.85 | Moderate synergism |
| 0.85–0.90 | Slight synergism |
| 0.90–1.10 | Nearly additive |
| 1.10–1.20 | Slight antagonism |
| 1.20–1.45 | Moderate antagonism |
| 1.45–3.30 | Antagonism |
| 3.30–10.0 | Strong antagonism |
| > 10.0 | Very strong antagonism |

## Results

### Alkaline Phosphatase Assay (ALP)

Ishikawa cells were incubated for 48 h with different concentrations ranging from 0.001 to 10 nM in case of ZEN and its metabolites and 0.001 to 10 µM in case of ISF, with 1:10 dilution steps in between. An exception was GLY due to its low estrogenicity and instead of 0.001 µM, 20 µM was included in the assessments. As positive control 1 nM E2 was used and 1% DMSO served as solvent control and their effects on ALP induction were set to 100% and 0%, respectively.

### ALP single substances

For the mycoestrogens, the most pronounced estrogenic effect was induced by α-ZEL followed by α-ZAL and ZEN with increasing concentrations (Fig. 4), while ZEN-14-S did not induce the ALP activity in the tested concentration range. Furthermore, in a concentration-dependent manner the tested ISF induced an estrogenic response up to a concentration of 1 µM with the highest impact of GEN, followed by EQ and DAI (Fig. 4). At a concentration of 10 µM, a decrease in ALP induction was observed for all three ISF. GLY showed only marginal estrogenic effects and a slight

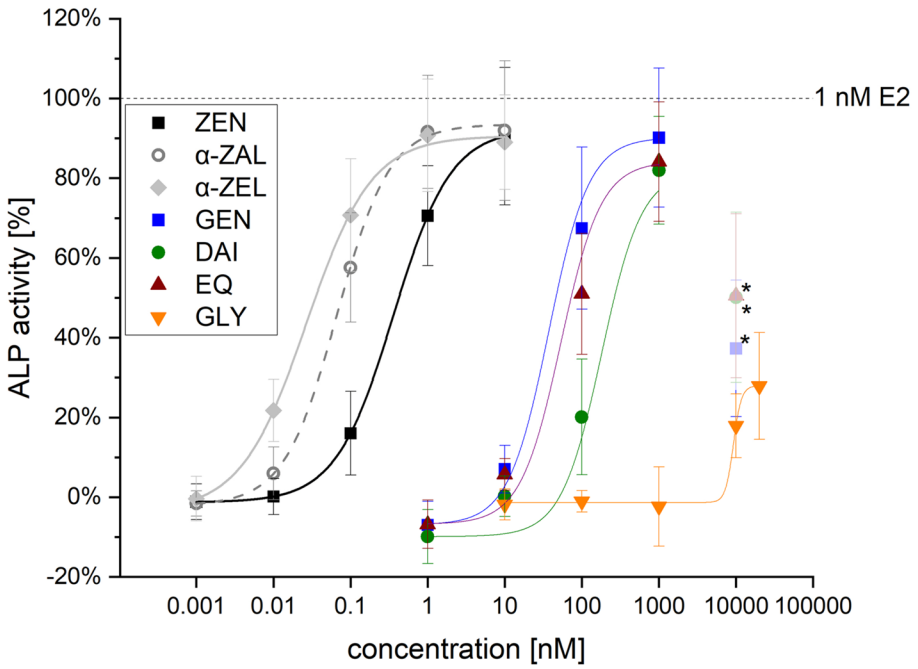


**Fig. 4** Dose–response curves of zearalenone (ZEN), α-zearalanol (α-ZAL), α-zearalenol (α-ZEL) and the isoflavones genistein (GEN), daidzein (DAI), equol (EQ) and glycitein (GLY). Sigmoidal-dose–response curve fits of estrogen-dependent activation of alkaline phosphatase activity (ALP) in Ishikawa cells after 48 h incubation. Results are depicted as mean ± standard deviation of at least 17 biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage). Outliers after the Nalimov outlier test as well as values marked with * were not included in the sigmoidal-dose-response curve fit. Effects of the solvent control (1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively

increase in ALP activity starting from 10 µM was observed which further increased at 20 µM.

The highest ALP activity was induced at a concentration of 10 nM by ZEN, α-ZEL and α-ZAL, which was about 90% compared to 1 nM E2. A concentration of 1 µM GEN, DAI and EQ showed the highest ALP induction of 82–90% related to 1 nM E2. In contrast, 20 µM GLY only reached 28 ± 13% of the ALP induction of 1 nM E2.

Sigmoidal dose–response curve fitting was performed for every single substance. By this analysis, the effective concentration that induces a 50% response ($EC_{50}$), a commonly used measure of toxin potency, can be determined. The obtained $EC_{50}$ values for the mycoestrogens were 0.027 ± 0.003 nM (α-ZEL), 0.067 ± 0.004 nM (α-ZAL) and 0.359 ± 0.001 nM (ZEN), and for the ISF 0.037 µM (GEN), 0.054 µM (EQ), 0.181 µM (DAI) and 9.25 µM (GLY). Since only the measured values for the lower four ISF concentrations were used for the $EC_{50}$ calculation, no standard deviation can be provided.

## ALP of combinations

All measured effects of single substances and combinations are compiled in the heatmaps (Figs. 5, 6, 7, S1). Results are expressed as a percentage of induction, where 0 and 100% represent the values of the solvent control (1% DMSO) and of 1 nM E2, respectively. Significant ALP activation of combinations compared to their respective single substances is indicated by "*x*" in the case of ISF and by "#" for mycoestrogens. The color code of these heatmaps indicates the strength of the effect, which enables a visual interpretation of the results of all tested combinations.

For all data sets, the combinations of ISF with ZEN, α-ZEL and α-ZAL showed similar trends, except for GLY. Effects were most pronounced at low to medium concentrations but decreased at higher doses. ISF concentrations in a range of 0.01–1 µM were found to mediate the most potent effects on the estrogenic activity of mycoestrogens. Except for GLY (Fig. S1), which potentiated the estrogenic effect of low mycoestrogen concentrations at higher levels (10–20 µM). A threshold to enhance the estrogenicity of mycoestrogens was found between 0.01 and 0.1 µM depending on the ISF (Figs. 5, 6, 7).

In binary mixtures with higher mycoestrogen concentrations (1 and 10 nM), GEN, DAI, and EQ only slightly enhanced the ALP induction compared to the mycoestrogens alone. Some combinations of ISF and mycoestrogens even exceeded the ALP activation of 1 nM E2 (values

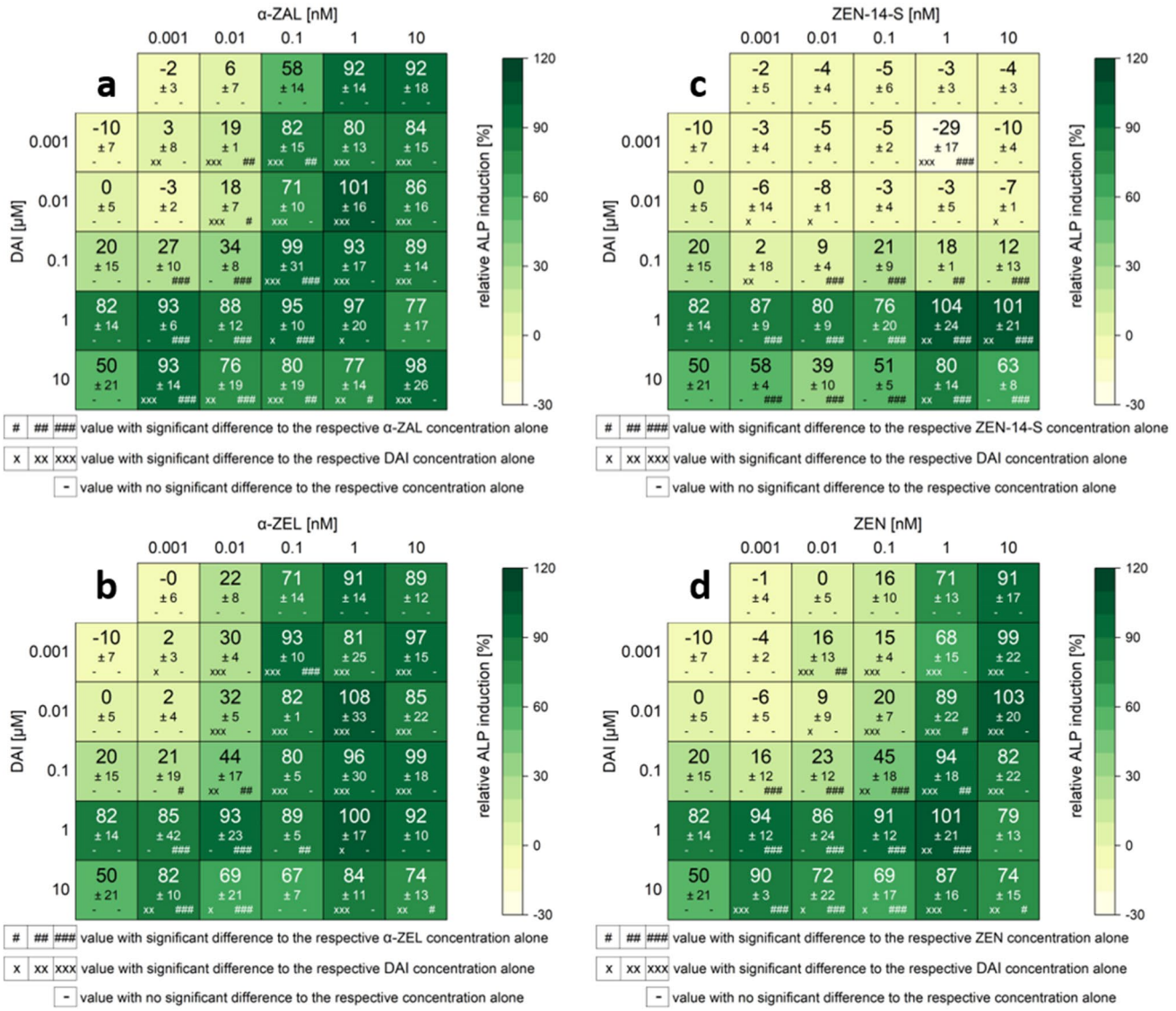


**Fig. 5** Effects of the combination of mycoestrogens with daidzein (DAI) on the ALP activity. Heatmaps indicating effects of single substances and combinations of α-zearalanol (α-ZAL) (**a**), α-zearalenol (α-ZEL) (**b**), zearalenone-14-sulfate (ZEN-14-S) (**c**) and zearalenone (ZEN) (**d**) with DAI on the alkaline phosphatase (ALP) activity in Ishikawa cells after 48 h incubation. Results are depicted as mean ± standard deviation of at least four biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage). Outliers after the Nalimov outlier test were excluded. Effects of the solvent control (1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively. The color code indicates the strength of the effects. Normal distribution of data was tested according to Shapiro–Wilk normality test and significance by one-way ANOVA. Significant differences in effects to the respective single substance concentration were indicated with $x = p < 0.05$, $xx = p < 0.01$ and $xxx = p < 0.001$ in the case of DAI and $\# = p < 0.05$, $\#\# = p < 0.01$ and $\#\#\# = p < 0.001$ in case of mycoestrogens."–" corresponds to no significant difference to the respective concentration of the single substance

above 100%). The highest ALP activity for combinations of mycoestrogens with GEN, DAI and EQ was mostly induced at a concentration of 1 µM for the ISF. Overall, the combination of EQ and ZEN showed the most potent estrogenic activities with an ALP induction of 115% (combination of 0.01 nM ZEN + 1 µM EQ) (Fig. 6d). In Fig. 8, selected combinations derived from the heat map of Fig. 6d are shown. The line graphs visualize the strength of the respective EQ concentrations in increasing the ALP activity. Additionally, EQ has the strongest effect to potentiate the estrogenic effect in mixtures with α-ZEL and α-ZAL compared to the other ISF.

The highest concentration of 10 µM ISF leads to a significant reduction in ALP activity by 32–53%. This effect was also seen in mixtures with mycoestrogens, albeit they suppressed the reduction. In total, in lower concentrations

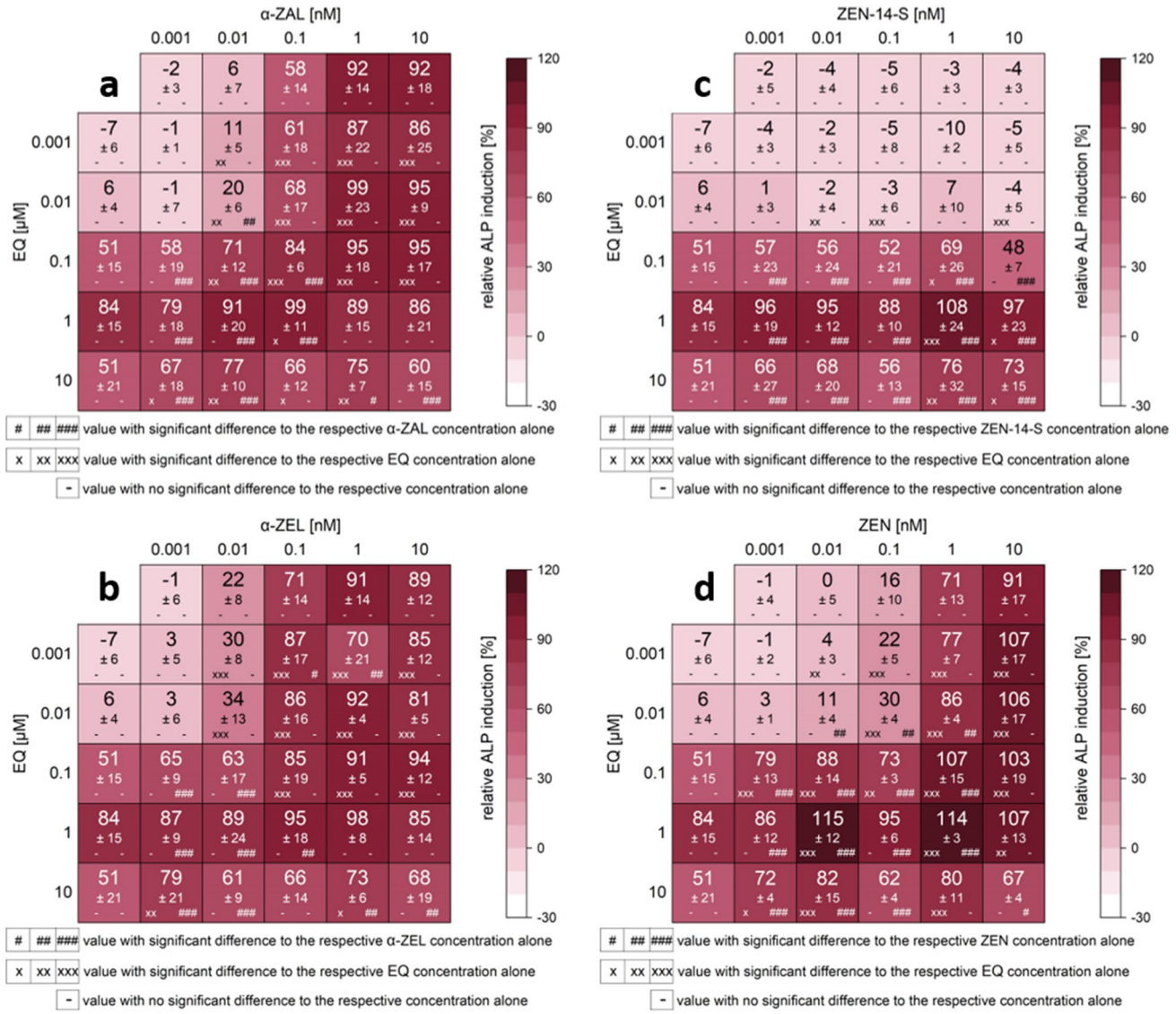


**Fig. 6** Effects of the combination of mycoestrogens with equol (EQ) on the ALP activity. Heatmaps indicating effects of single substances and combinations of α-zearalanol (α-ZAL) (**a**), α-zearalenol (α-ZEL) (**b**), zearalenone-14-sulfate (ZEN-14-S) (**c**) and zearalenone (ZEN) (**d**) with EQ on the alkaline phosphatase (ALP) activity in Ishikawa cells after 48 h incubation. Results are depicted as mean ± standard deviation of at least four biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage). Outliers after the Nalimov outlier test were excluded. Effects of the solvent control (1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively. The color code indicates the strength of the effects. Normal distribution of data was tested according to Shapiro–Wilk normality test and significance by one-way ANOVA. Significant differences in effects on the respective single substance concentration were indicated with $x = p < 0.05$, $xx = p < 0.01$ and $xxx = p < 0.001$ in the case of EQ and $\# = p < 0.05$, $\#\# = p < 0.01$ and $\#\#\# = p < 0.001$ in case of mycoestrogens. "−"corresponds to no significant difference in the respective concentration of the single substance

than 10 µM, GEN, DAI and EQ showed enhancing estrogenic effects in binary mixtures with mycoestrogens. Mixtures of GLY with mycoestrogens showed different results in the applied system. Concentrations up to 1 µM GLY did not lead to any estrogenic effect. This was also seen for most combinations with mycoestrogens. In the concentration range of 0.01 to 1 µM, GLY did not increase the ALP activity of the mycoestrogens compared to their respective single substances. Only starting from 10 µM, GLY showed estrogenic effects. Interestingly, low levels of mycoestrogens (0.001–0.1 nM) increased the ALP activity. In combination with 1 and 10 nM mycoestrogens, the ALP induction was decreased compared to mycoestrogens alone.

The estrogenic effects of the phase II metabolite of ZEN, namely ZEN-14-S, were different compared to ZEN and

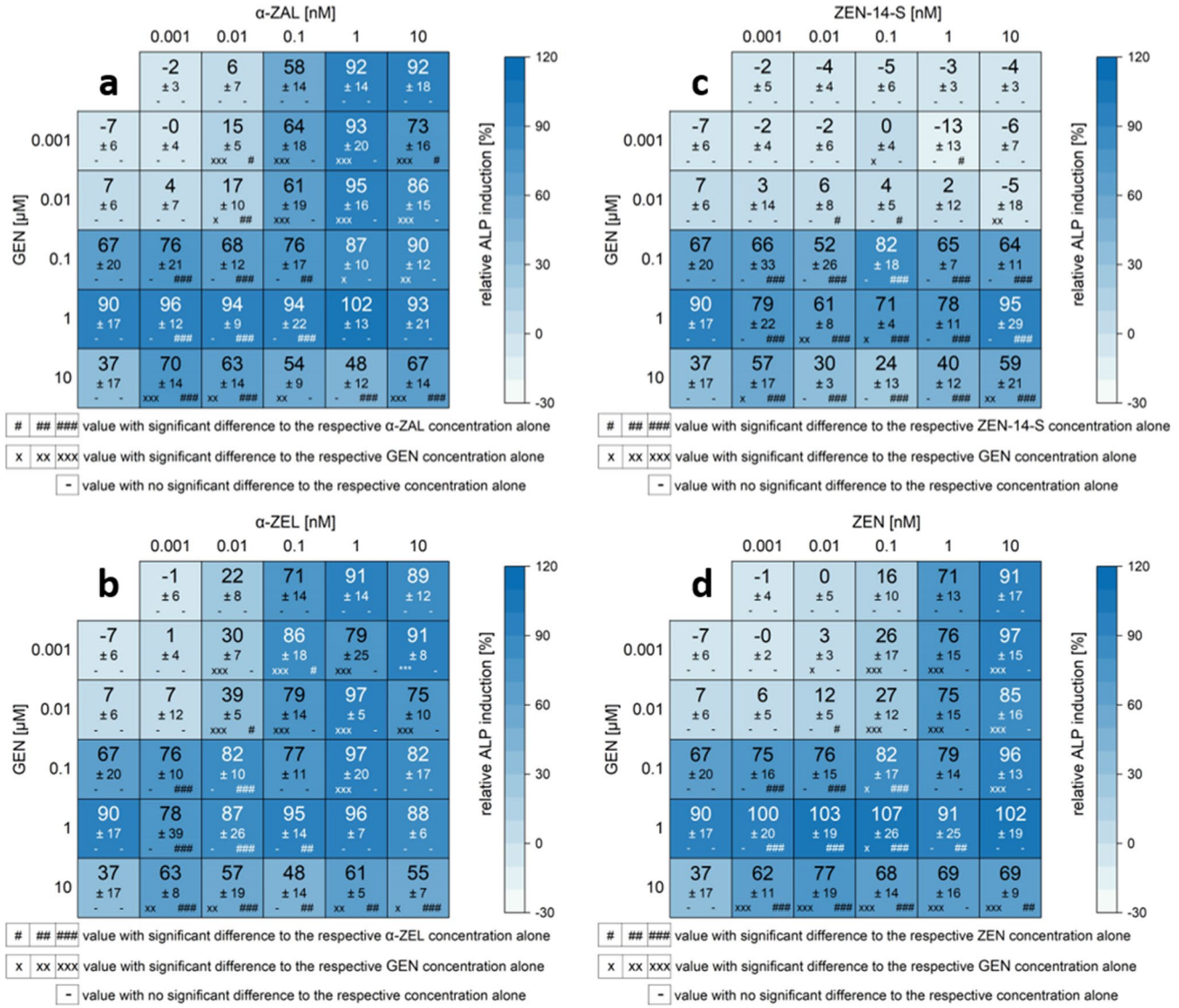


**Fig. 7** Effects of the combination of mycoestrogens with genistein (GEN) on the ALP activity. Heatmaps indicating effects of single substances and combinations of α-zearalanol (α-ZAL) (**a**), α-zearalenol (α-ZEL) (**b**), zearalenone-14-sulfate (ZEN-14-S) (**c**) and zearalenone (ZEN) (**d**) with GEN on the alkaline phosphatase (ALP) activity in Ishikawa cells after 48 h incubation. Results are depicted as mean ± standard deviation of at least four biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage). Outliers after Nalimov outlier test were excluded. Effects of the solvent control (1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively. The color code indicates the strength of the effects. Normal distribution of data was tested according to Shapiro–Wilk normality test and significance by one-way ANOVA. Significant differences in effects on the respective single substance concentration were indicated with $x = p < 0.05$, $xx = p < 0.01$ and $xxx = p < 0.001$ in case of GEN and $\# = p < 0.05$, $\#\# = p < 0.01$ and $\#\#\# = p < 0.001$ in case of mycoestrogens. "–"corresponds to no significant difference in the respective concentration of the single substance

its phase I metabolites. No induction of ALP activity was observed in the applied concentrations. In combination with low levels of ISF (0.001–0.1 nM), no stimulatory action was observed. However, higher ISF concentrations (1–10 µM) mixed with ZEN-14-S significantly increased the ALP induction in some cases.

## Cytotoxicity

Cytotoxicity of single substances and combinations was measured with the CTB assay to assess the metabolic activity and the SRB assay to assess the protein content. This was done to exclude the generation of artefacts due to cytotoxicity or induced cell growth.

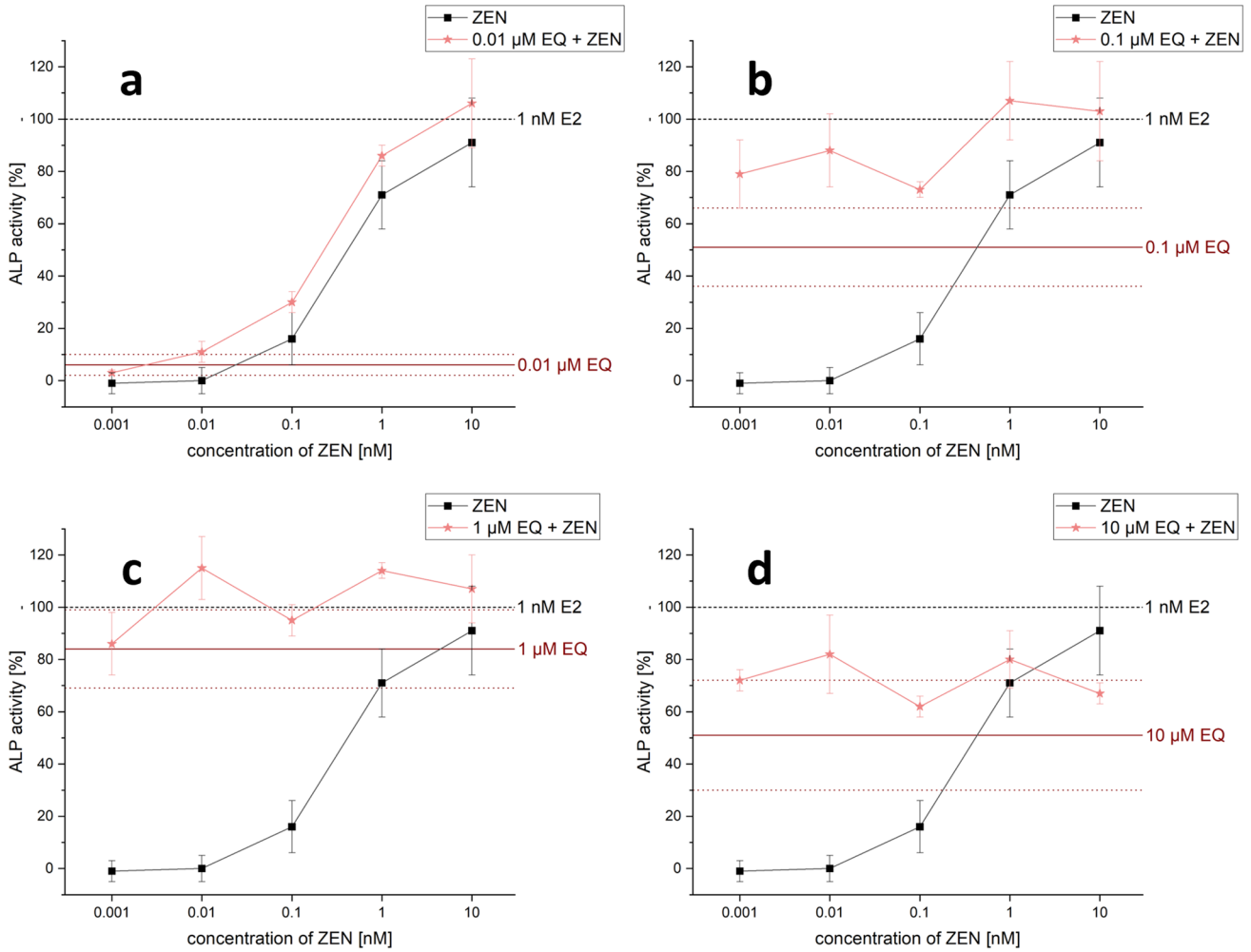


**Fig. 8** Selected diagrams for the combinatory estrogenic effect of zearalenone (ZEN) and equol (EQ). Illustration of the combinatorial estrogenic effects of ZEN and EQ on the basis of increasing ZEN concentrations and fixed EQ concentrations on the alkaline phosphatase (ALP) activity in Ishikawa cells after 48 h incubation. Results are depicted as mean and standard deviation of at least four biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage). The fixed EQ concentration is displayed as a red line and the corresponding standard deviation as dashed red lines. Results were referred to 1 nM E2 as positive control set to 100% and the solvent control (1% DMSO) set to 0%

## ZEN and its metabolites

No concentration-dependent cytotoxicity could be determined in the tested concentration range of ZEN, α-ZEL, α-ZAL and ZEN-14-S, neither by the CTB nor by the SRB assay (Fig. 9a, b). However, a slight significant increase in cell viability and protein quantity was determined between test substances and solvent control for some mycoestrogens in higher concentrations. Only α-ZAL in the concentration of 0.001 and 0.01 nM lead to a decreased protein content in the SRB assay. Since α-ZAL showed no decreased protein amount at higher concentrations, this effect was probably not due to cytotoxicity, but due to losses during the processing steps.

## Isoflavones

No significant decrease in metabolic activity (Fig. 10a) or in the cell protein amount (Fig. 10b) was observed for GEN, DAI and EQ. In contrast GEN, DAI and EQ showed a significant tendency in increasing the metabolic activity (Fig. 10a) and cell protein amount (Fig. 10b) at certain concentrations. Concentrations of 10 and 20 µM GLY lead to a slight significant decrease in cell viability and cell protein amount.

## Cytotoxicity of combinations

As shown in Fig. 11, the metabolic activity (CTB assay) and the protein content (SRB assay) of the two highest concentrated combinations between ISF and ZEN and its

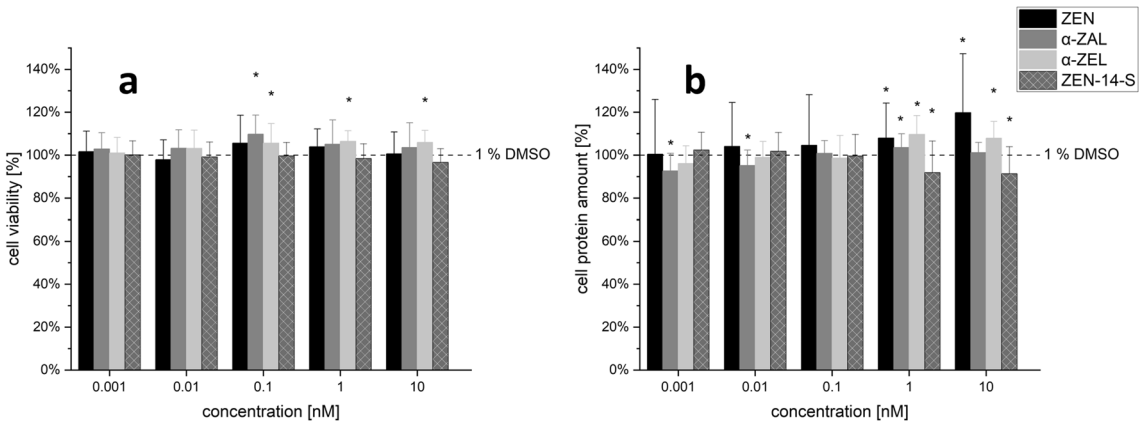


**Fig. 9** Effects of zearalenone (ZEN), α-zearalanol (α-ZAL), α-zearalenol (α-ZEL) and ZEN-14-sulfate (ZEN-14-S) as single substances on the cytotoxicity. Impact on the cell viability [%] measured by the CellTiter Blue (CTB) assay (**a**) and on the cell protein amount [%] measured by the sulforhodamine B (SRB) assay (**b**) of increasing concentrations of ZEN and its metabolites after 48 h incubation in Ishikawa cells. Values were referred to the solvent control (1% DMSO) set to 100%. Results are depicted as mean ± standard deviation of at least 5 biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage). Outliers after the Nalimov outlier test were excluded. Significant differences of effects between the solvent control and the incubation solutions were calculated by one-sample Student's *t*-test and significances are indicated with $*p < 0.05$

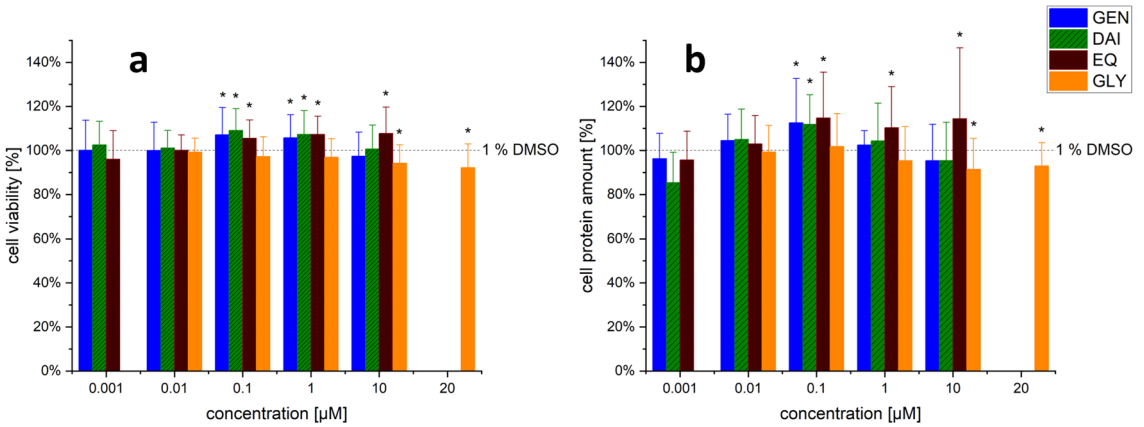


**Fig. 10** Effects of genistein (GEN), daidzein (DAI), equol (EQ) and glycitein (GLY) on cell viability. Impact on the cell viability [%] measured by the CellTiter Blue (CTB) assay (**a**) and on the cell protein amount [%] measured by the sulforhodamine B (SRB) assay (**b**) of rising concentrations after 48 h incubation in Ishikawa cells. Values were referred to the solvent control (1% DMSO) as 100%. Results are depicted as mean + standard deviation of at least 20 biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage). Outliers after the Nalimov outlier test were excluded. Significant differences in effects between the solvent control and the incubation solutions were calculated by one-sample Student's *t*-test. Significances are indicated with $*(p < 0.05)$. According to its low estrogenicity, GLY was tested in one higher concentration (20 µM) instead of the lowest concentration of 0.001 µM

metabolites are shown exemplarily. No significant decrease in metabolic activity or protein content was induced. However, for some combinations a significant increase in the two applied systems was generated. Furthermore, cytotoxic effects were also assessed in lower concentrations (Supplement Information Figs. S2–S5).

## Combination Index (CI)

Some requirements have to be fulfilled for the assessment of the CI. Therefore, no CI calculation was possible for ZEN-14-S and GLY as they had no or only a minor impact on the ALP activity at the applied concentrations. Only the CI values of combinations between GEN, DAI and EQ together with ZEN, α-ZEL and α-ZAL were evaluated. Furthermore, only values between zero and one can be considered. As


**Fig. 11** Effects of the combination of mycoestrogens (zearalenone (ZEN), α-zearalanol (α-ZAL), α-zearalenol (α-ZEL) and ZEN-14-sulfate (ZEN-14-S)) with the isoflavones (genistein (GEN), daidzein (DAI), equol (EQ) and glycitein (GLY)) on the cytotoxicity. Impact on the cell viability [%] measured by the CellTiter Blue (CTB) assay and on the cell protein amount [%] measured by the sulforhodamine B (SRB) assay of single substances and combinations of the highest concentrations of ZEN and its metabolites (1 nM and 10 nM) with the highest concentrations of isoflavones (1 µM and 10 µM for GEN, DAI, and EQ or 10 µM and 20 µM for GLY) after 48 h in Ishikawa cells. Values were referred to the solvent control (1% DMSO) as 100%. Results are depicted as mean ± standard deviation of at least four biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage). Outliers after the Nalimov outlier test were excluded. Significant differences in effects between the solvent control and the incubation solutions were calculated by one-sample Student's *t*-test. Significances are indicated with # ($p < 0.05$)

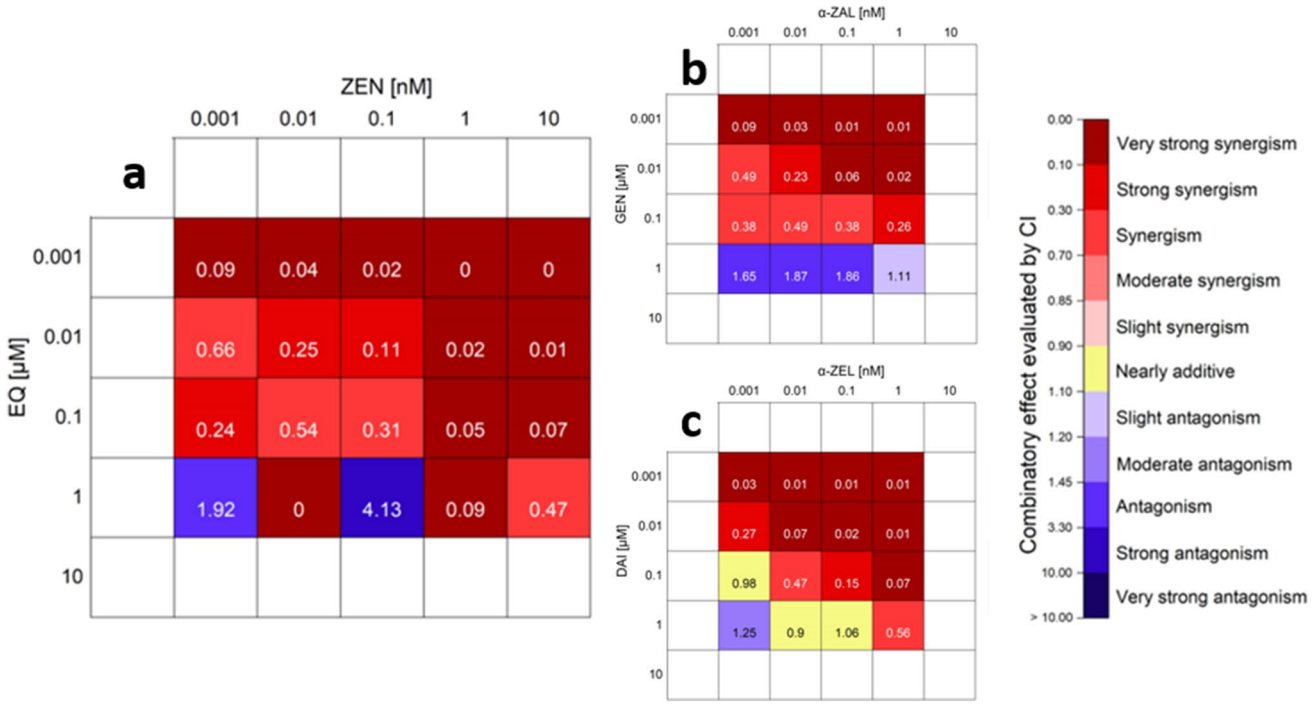


**Fig. 12** Combination index (CI) of selected combinations of mycoestrogens and isoflavones. Heatmaps indicating the CI of the combination zearalenone (ZEN) with equol (EQ) (**a**), α-zearalanol (α-ZAL) with genistein (GEN) (**b**) and α-zearalenol (α-ZEL) with daidzein (DAI) (**c**) of the estrogenic action measured by alkaline phosphatase (ALP) activity and calculated according to Chou (Chou 2006). The CI was only calculated for the measured values which fulfilled the requirements for calculation. The color code indicates the combinatory effect of the mixture. Red corresponds to values < 0.9 and therefore synergism, yellow for CI between 0.9 and 1.1 for nearly additive effects and blue for antagonism with CI values of > 1.1. In addition, the statistical analysis of the measured dataset obtained from the ALP measurements is present

in some cases, the ALP inductions were lower than 0% or higher than 100%, the effects had to be recalculated and related to -26% and 115% which corresponds to the lowest and highest ALP activity of all single substances and combinations thereof. Additionally, only values which are in the linear range of the dose–response curve may be considered. Thus, 10 µM GEN, DAI and EQ and 10 nM α-ZEL and α-ZAL as single substances were not considered for the CI calculation. We calculated the CI for the combinations α-ZEL with DAI, ZEN with EQ and α-ZAL with GEN to provide a representative for every single substance (see Fig. 12).

For all combinations, the incubation of 0.001 µM ISF with various mycoestrogen concentrations had a very strong synergistic effect on the ALP induction. At ISF concentrations of 0.01 and 0.1 µM, the combinatory effects were strongly synergistic or synergistic. However, at an ISF concentration of 1 µM in combinations with various mycoestrogen concentrations, the combinatory effects varied. For the mixtures between α-ZEL with DAI (1 µM), respectively, the CI values indicate an additive or even antagonistic effect, whereas the combination of ZEN and EQ (1 µM) showed strong to very strong synergism or antagonism.

## Discussion

Consumers are exposed to a wide range of undesirable and partly toxic compounds at the same time. Therefore, not only the individual effect of one substance should be evaluated, but also an interaction with other toxins or bioactive agents should be considered to provide a reliable risk assessment. Previous studies have shown that xenoestrogens can potentiate their toxic effect in vitro (Vejdovszky et al. 2017a, b). As recently reviewed, mycoestrogens and phytoestrogens co-occur together frequently in various animal feed commodities (Grgic et al. 2021). We decided to investigate the estrogenic effect of the three major ISF (GEN, DAI and GLY) found in soy and the gut microbial metabolite of DAI EQ, as soy is one of the most frequently used protein sources in animal feed. On the other hand, corn is the major carbohydrate additive in animal feed. One of the most common mycotoxins found in corn is ZEN with an incidence of 81% (Munkvold et al. 2018).

ISF and ZEN are known to exhibit estrogenic properties. However, as ISF occur in higher concentrations and 1000 times higher concentrations are required to induce estrogenic stimuli compared to ZEN and its metabolites, we investigated the combinatory effects in concentration ratios of 1:1—1000:1 (ISF:mycoestrogen) in steps with a factor of 10 in between. This demonstrates the importance to assess their combinatory effects especially when the same mechanism is addressed. Therefore, our aim was to investigate the potential of ISF combined with ZEN and its metabolites on a possible increase of estrogenic effects in vitro.

Measurements of single mycotoxins (Fig. 4) confirmed previous reports that α-ZEL is the most potent estrogen followed by α-ZAL and the parent compound ZEN determined in human MCF-7 (Molina-Molina et al. 2014) and Ishikawa cells (Vejdovszky et al. 2017a; Mendez-Catala 2020). In contrast, ZEN-14-S seems to possess no estrogenic potential due to conjugation. This loss of estrogenic activity after the conjugation at C14 of ZEN was already determined for ZEN-14-glucose in silico experiments (Dellafiora et al. 2017). Under the applied conditions, ZEN-14-S did not seem to deconjugate to ZEN as no increase in ALP activity was observed.

ZEN is able to induce cytotoxic effects at very high concentrations (39.3–78.5 mM; 39.7 ± 9.6 µM) in K 562 (Reubel et al. 1987) and HepG2 cells, respectively (Marin et al. 2019). However, at the applied concentrations of this study (0.001 to 10 nM), no concentration-dependent cytotoxicity could be determined for ZEN, α-ZEL, α-ZAL and ZEN-14-S by assessing the metabolic activity or the protein content in the Ishikawa cells (Fig. 9). On the contrary, some concentrations lead to significant increases of the measured parameters. It can be hypothesized that ZEN or its metabolites may trigger mitochondrial swelling and possibly lead to the formation of megamitochondria which could at least partially lead to increased metabolic activity. This effect has already been hypothesized for deoxynivalenol (Krishnaswamy et al. 2010; Springler et al. 2017). This result indicates that mycoestrogens might have proliferative effects, although the CTB and SRB were only performed to investigate the cytotoxic effect to rule out that a reduced ALP activity is caused by cytotoxicity. Longer incubations than the applied 48 h would have been required to clearly assess proliferative effects, but this is not the focus of the present article.

Especially in soy-containing products and finished feed, ISF usually occur in higher (100–1000 times) concentrations compared to mycoestrogens (Lee et al. 2003; Grgic et al. 2021). Therefore, ISF are consumed in higher amounts by mammals with plasma concentrations reaching up to a low two-digit µM range (Vitale et al. 2013). Thus, a wide range up to a concentration of 20 µM ISF was tested to assess the estrogenic potency. As seen in Fig. 4, the induction of ALP expression by GEN, DAI and EQ increased concentration-dependently, except for the highest tested concentration (10 µM). In case of GLY only the two highest applied concentrations (10 and 20 µM) enhanced ALP expression and only to a limited percentage compared to 1 nM E2 (by 18 ± 8% and 28 ± 13%, respectively). Compared to ZEN and its metabolites, 100 to 1000 times higher concentrations of ISF were required to reach the same estrogenic impact. Taken together, in Ishikawa cells the estrogenic potential of ISF can be ranked as GEN > EQ > DAI > > > GLY.

Other studies indicate that EQ has the highest affinity to bind to ER using human estrogen receptor (hER) transfected *Saccharomyces cerevisiae* and MCF-7 cells (Morito et al. 2001; Mortensen et al. 2009). However, the ALP induction in Ishikawa cells after GEN and EQ incubation hardly differ and estrogenic effects may vary between cell lines, applied assay system and measured endpoint. Furthermore, GEN and EQ are ranked by the United Kingdom Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) to possess the highest estrogenic potential of all ISF (Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment 2011).

In the case of GEN, DAI and EQ the highest applied concentration (10 µM) lead to a minor induction of the ALP compared to 1 µM. This decreased ALP activity for 10 µM ISF cannot be fully explained by a slight cytotoxic effect but might arise from an overlay of different cellular mechanisms. In the case of GEN, a spectrum of cellular mechanisms in different concentration ranges have been reported, including inhibition of protein kinases (Chang and Geahlen 1992; Kurzer and Xu 1997). Phosphorylation steps represent important signals in the ER pathway, potentiating ER genomic signaling activity on gene transcription (Arpino et al. 2008). Therefore, it might be speculated that inhibitory effects on respective kinases are involved in the apparent suppression of estrogenic response in the highest applied concentration of GEN, DAI and EQ.

A further possible explanation could be a suppression of estrogen receptor expression. This has already been shown in in vivo experiments, where high doses of ISF were able to decrease *ESR1* (gene encoding estrogen receptor α) mRNA levels in rat uterus (Cotroneo et al. 2001). Furthermore, higher ISF concentrations are able to induce the transcription of cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*), which in turn leads to increased metabolism of xenoestrogens and might be another explanation for the reduced estrogenicity at the highest concentration (Satih et al. 2010; Wei et al. 2015).

Phase II metabolism of ISF may play a further role in the reduced estrogenic activity. In humans mainly sulfoglucuronides and diglucuronides circulate in the human body (Hosoda et al. 2011; Soukup et al. 2016). This was

also observed for ISF in endothelial cells where the glucuronide and sulfate conjugates are predominant (Toro-Funes et al. 2015). This conjugation is considered to detoxify the isoflavones, which might lead to reduced estrogenic effects (Setchell et al. 1987). However, there is also data that indicates that phase II metabolites of ISF are still biologically active, albeit to a much lower extent than their parent compounds (Hüser et al. 2018; Pugazhendhi et al., 2008). This could be a further possible explanation for the reduced estrogenic activity of ISF at the highest concentration.

Regarding combinatory effects between mycoestrogens and ISF, the results demonstrated that for most combinations higher ALP inductions were achieved compared to the respective single substances (Figs. 5, 6, 7). Taken together, the results of the CI calculation indicate synergistic estrogenic effects between ZEN, α-ZEL, α-ZAL and GEN, DAI, EQ. These effects were most pronounced at lower concentrations of mycoestrogens and ISF. With increasing ISF concentrations (10 µM), the interaction shifted towards additive or even antagonistic effects (Fig. 12). At this ISF concentration (10 µM), in some cases, the addition of medium to high concentrations of mycoestrogens led to a lower induction of ALP activity compared to the respective single substances. A possible explanation for this effect might be that ISF in these high concentrations are saturating and binding to both ER and therefore, the mycoestrogen is not able to exert its estrogenic potential (Nikov et al. 2000). In general, the combinations of the parent mycotoxin ZEN with ISF showed the most potent estrogenic stimulus, which was reflected by the respective CI (Fig. 12). This was unexpected as the phase I metabolites α-ZEL and α-ZAL as single compounds had stronger estrogenic effects (Fig. 4), suggesting that their estrogenic impact might arise also in combination. The most prominent enhancing effect was observed with the combination of ZEN and EQ where an ALP induction of up to 115 ± 12% was reached (Fig. 6d, 12a). However, α-ZEL and α-ZAL in mixtures with ISF also increased the ALP expression for some combinations significantly (Figs. 5, 6, 7). Out of all ISF, GLY showed the lowest estrogenic potential as a single substance, which was also seen in combinations with mycoestrogens (Fig. S1).

We hypothesized that the enhanced estrogenic effects of combinations between phyto- and mycoestrogens are based on the interaction of the respective substances with either ERα or ERβ, which are both expressed by Ishikawa cells. While ZEN and its metabolites have a stronger affinity to bind to both ER, ISF preferably interact with ERβ (Nikov et al. 2000; Setchell et al. 2005; Takemura et al. 2007). This has already been elucidated for ZEN, GEN and EQ for the human ERα and ERβ. ZEN shows a similar relative binding affinity (RBA) to ERα and ERβ, which is 8 and 11, respectively. In contrast, the RBA for GEN to bind to ERα and ERβ is dissimilar. Its RBA to ERβ is significantly higher (RBA = 31) compared to binding to ERα (RBA = 1). This observation was also seen for EQ, where its affinity increases from 0.3 for ERα to 3 for ERβ.

ZEN-14-S as a phase II metabolite is classified as a masked mycotoxin and might be hydrolyzed to ZEN after entering the colon through the enterohepatic cycle. Furthermore, there were no current studies on the estrogenic potential of ZEN-14-S. In contrast to the phase I metabolites, ZEN-14-S had no effects on ALP activity as a single substance in the applied system, so enhancing estrogenic effects in combinations with ISF were not expected. Only in some cases, high ISF concentrations lead to a slightly increased trend in ALP activity. The conjugation of ZEN with glucuronides has already been described as a detoxification process (Dellafiora et al. 2017). The sulfate conjugate showed no estrogenic properties neither as a single substance nor in combination with ISF and is therefore considered as a detoxifying metabolite.

For GEN, DAI and EQ as single compounds, the onset of estrogenic response is observed at a threshold value between 0.01 and 0.1 µM. Several studies indicated that in populations with high soy consumption the ISF blood plasma levels can reach 0.1 to 0.9 µM (Gooderham et al. 1996; Verkasalo et al. 2001). In this concentration range, GEN, DAI and EQ already mediate estrogenic effects. However, in mixtures with mycoestrogens these low concentrations are able to potentiate the estrogenic effects. In farm animals even higher ISF plasma concentrations can be detected between 1 and 10 µM (Grgic et al. 2021). In these high concentrations combined with mycoestrogens even higher ALP activities were induced compared to 1 nM E2. In contrast, in tissue and cells where only one ER is predominant, like it is the case for estrogen-sensitive breast cancer cells (ERα) no enhanced estrogenic effects are expected. On the contrary, high ISF concentrations (> 1 µM) could suppress estrogenic effects which are induced by mycoestrogens. Our study demonstrates the interactive effects of phyto- and mycoestrogens, which would require verification in in vivo studies to clarify the consumer´s risk. However, it is necessary that not only the toxicity of single substances is considered for risk assessments, but also combinatory effects of realistic uptake scenarios to ensure consumer's safety. This in fact would indicate that the maximal tolerable amount of ZEN in various food and feed stuff should be reconsidered and subsequently adopted, as synergistic effects may increase the hazard. Furthermore, the risk assessment for phytoestrogens should be discussed with regard to the high exposure from feed and resulting possible negative effects affecting the reproductive system of certain farm animals.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00204-022-03358-3.

**Author Contributions** Conceptualization: DG, EV, BN and DM; Methodology: DG, EV and DM; Formal analysis and investigation: DG, AB and RF; Writing—original draft preparation: DG; Writing—review and editing: AB, RF, EV, BN and DM; Funding acquisition: EV, BN and DM; Resources: BN, DM; Supervision: EV, BN and DM.

**Funding** Open access funding provided by University of Vienna. This research was funded by the Austrian Research Promotion Agency (FFG) and BIOMIN Holding GmbH through the Bridge project "ISOMYCOTOX—Combinatory endocrine activity of mycoestrogens and soy isoflavones in porcine feed" (project number 880656).

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.



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## 8.3 Publication 3:

“Estrogen receptor α interaction of zearalenone and its phase I metabolite α-zearalenol in combination with soy isoflavones in hERα-HeLa-9903 cells”

Mycotoxin Research
https://doi.org/10.1007/s12550-023-00506-1

ORIGINAL ARTICLE

# Estrogen receptor α interaction of zearalenone and its phase I metabolite α-zearalenol in combination with soy isoflavones in hERα-HeLa-9903 cells

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Received: 29 July 2023 / Revised: 10 September 2023 / Accepted: 5 October 2023


## Abstract

Risk assessment primarily relies on toxicological data of individual substances, with limited information on combined effects. Recent in vitro experiments using Ishikawa cells, an endometrial carcinoma cell line expressing both estrogen receptor isoforms, demonstrated interactive effects of phyto- and mycoestrogens. The mycoestrogens, zearalenone (ZEN), and α-zearalenol (α-ZEL) exhibited significantly enhanced estrogenic responses in the presence of isoflavones (ISF), depending on substance ratios and concentrations. This study investigated the impact of phyto- and mycoestrogen combinations on estrogenic response following OECD guideline 455, utilizing hERα-HeLa-9903 cells. Test substances included mycoestrogens (ZEN and α-ZEL) and isoflavones (genistein (GEN), daidzein (DAI), and S-equol (EQ), a gut microbial metabolite of DAI). Mycoestrogens were tested in the range of 0.001 to 100 nM, while isoflavones were used at concentrations 1000 times higher based on relevant occurrence ratios. Results showed that ZEN and α-ZEL induced ERα-dependent luciferase expression in concentrations above 1 nM and 0.01 nM, respectively. However, ISF caused a superinduction of the luciferase signal above 1 µM. A superinduction is characterized by an unusually strong or heightened increase in the activity of the luciferase enzyme. This signal is not affected by the estrogen receptor antagonist 4-hydroxytamoxifen (4-OH-TAM), which was additionally used to verify whether the increase of signal is a true reflection of receptor activation. This superinduction was observed in all combinations of ZEN and α-ZEL with ISFs. Contrary to the luciferase activity findings, RT-qPCR experiments and a stability approach revealed lower real ERα activation by ISFs than measured in the ONE-Glo™ luciferase test system. In conclusion, the OECD protocol 455 appears unsuitable for testing ISFs due to their superinduction of luciferase and interactions with the test system, resulting in experimental artifacts. Further studies are necessary to explore structure–activity relationships within polyphenols and clarify the test system's applicability.

**Keywords** Isoflavones · Mycoestrogens · Combinatory toxicology · Risk assessment · Endocrine disruptors

## Introduction

The quintessence of toxicology imparts the usage of tools that helps us to understand the harmful effects anthropogenic or natural compounds can have on people, animals, and the environment. Moreover, it comprises the idea of every compound owning the capacity to act adversely, indifferent of their origin, yet depended on the inflicted dosage. New assessments become necessary, as studying the toxicity of single compounds portrays an unrealistic scenario of exposure, hence limiting the predictive powers of such investigations (Bates et al. 2018). So far, risk assessments are still predominantly based on the toxicological data of single substances, which often underestimate the toxicological potential of certain mixtures. Data on combinatorial effects are

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Published online: 17 October 2023

still scarce, and therefore, in order to ensure extensive health protection and food safety, further studies are required, leading to governmental authorities calling for a need to assess combinatory effects of chemical mixtures (Hartemann and Henstein 2011). Since the inclusion of mathematical methods which dates back to the early 1900s, where Loewe and Munischnek formulated a concept concerning the additivity of chemicals, the assessment of combinatory toxicology gained in popularity (Loewe and Munischnek 1926). Nowadays, newer models such as the Chou and Talalay's method and the CISNE approach by Garcia-Fuenta et al. have contributed to a further progress of this topic (Chou and Talalay 1983; García-Fuente et al. 2018).

Recently, interactive effects of phyto- and mycoestrogens were demonstrated in Ishikawa cells using the Chou and Talalay's method to calculate its combinatory effects (Grgic et al. 2022). Zearalenone (ZEN, Fig. 1) is a well-known mycoestrogen formed by *Fusarium* spp. Upon reductive metabolism, α-zearalenol (α-ZEL) is formed, a step which strongly enhances the estrogenic properties. ZEN is regulated by the European Food Safety Authority (EFSA) for certain animal feed and food stuff, but, for α-ZEL no obligatory limits are applied. However, these fungal metabolites frequently co-occur with non-regulated phytoestrogens as recently summarized for animal feed (Grgic et al. 2021).

In Ishikawa cells, expressing ERα and ERβ, low concentrations of ZEN, α-ZEL and α-zearalanol (0.001–0.01 nM) significantly enhanced the estrogenic response in combination with low concentrations of genistein (GEN), daidzein (DAI) or its gut microbial metabolite S-equol (EQ) (0.001–0.1 µM) compared to their respective single substances (Grgic et al. 2022).

The estrogenic effects of both ISF and ZEN and several of its metabolites are based on the structural and functional similarity to the endogenous hormone 17-β-estradiol (E2). The proposed mechanism of the synergistic estrogenic properties of combinations between ISF and ZEN and its metabolites is based on the different binding affinity of the respective compounds to both isoforms of the estrogen receptor (ERα and ERβ) (Grgic et al. 2022). ISF are well known to preferably bind to the ERβ, whereas ZEN and its metabolites have a high affinity to both isoforms (Nikov et al. 2000; Setchell et al. 2005; Takemura et al. 2007). Therefore, we hypothesize that, in hormone-sensitive cells, synergistic estrogenic effects between phytoestrogens and mycoestrogens in naturally occurring mixtures are mainly due to the presence of both ERs, whereas we do not expect to have synergistic effects when only ERα is present. Our aim in the present study was to demonstrate that synergistic effects of mycoestrogens and ISF are based on the presence of both ER (ERα and ERβ). Using the hERα-HeLa-9903 cells, which solely expresses ERα, synergistic effects were not expected but had to be proven. This cell line was selected according to the Guideline 455 of the Organisation for Economic Co-operation and Development (OECD) (OECD 2015) and the applicability of this guideline for the tested substances is investigated.

## Materials and methods

### Materials

Cell culture flasks and 96-well plates were purchased from Sarstedt (Nürnbrecht, Germany). Cell culture media (Dulbecco's Minimal Essential Medium (DMEM) and Eagle's Minimal Essential Medium (EMEM) without phenol red) and supplements (fetal bovine serum (FBS), charcoal–dextran stripped FBS (CD-FBS), blasticidin S HCl and geneticin (G418)) were produced from Gibco and obtained from Thermo Fisher Scientific (Waltham/MA, USA). ZEN, α-ZEL, E2, (Z)-4-hydroxytamoxifen (4-OH-TAM) and sulforhodamine B (SRB) were purchased

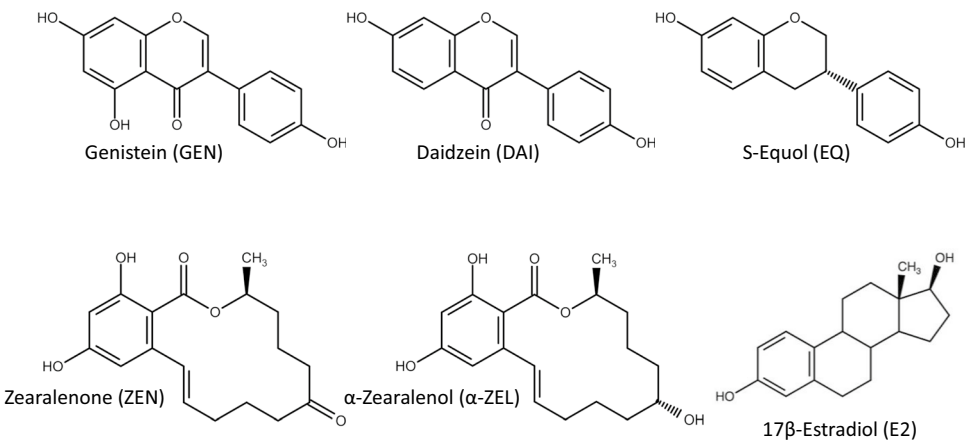


**Fig. 1** Chemical structures (created with: chemspace.com) of the investigated compounds—the isoflavones genistein (GEN), daidzein (DAI), S-equol (EQ), mycotoxins zearalenone (ZEN), α-zearalenol (α-ZEL), and the hormone 17-β-estradiol (E2)

from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). DAI, EQ, and GEN were obtained from Extrasynthese (Genay Cedex, France) whereas dimethly sulfoxide (DMSO), NaCl, KCl, $Na_2HPO_4$, $Na_2HPO_4$ * 2 $H_2O$, and $KH_2PO_4$ were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). The CellTiter-Blue® Cell Viability Assay Kit and ONE-Glo™ EX luciferase assay system were obtained from Promega Corporation (Madison/WI, USA).

## Cell line

The modified human cervical cell line for the identification of ERα agonists and antagonists also known as "hERα-HeLa-9903" was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Cell stocks were stored in liquid nitrogen containers and 2 weeks prior to the start of the in vitro experiments, cells were taken in culture. They were cultivated in an incubator at 37 °C with 5% $CO_2$ and 95% humidity using the growth medium consisting of DMEM, supplemented with 5% (*v/v*) heat-inactivated FBS, 16 µg/mL blasticidin S HCl and 800 µg/mL G418. Cells of the passage number 5 were split at a confluency of about 80% and kept in culture up to the maximum passage number of 40. Before the assays were performed, the growth medium was aspired and replaced with the assay medium consisting of EMEM supplemented with 5% CD-FBS.

## ONE-Glo™ EX luciferase (OGL) assay

The assays were performed in 96-well plates following the instructions of the manufacturer (Promega 2007). Per well, 10,000 hERα-HeLa-9903 cells in assay medium were seeded and grown for 3 h. Thereafter, the assay medium was replaced with the incubation solutions consisting of various concentrations of ZEN, α-ZEL, GEN, DAI, and EQ as single substances or in the respective combinations in assay medium. The substances were previously dissolved in DMSO in 2000 times higher concentrations than the tested concentration, followed by dilution in the assay medium. In case of single substance testing and for the solvent control, DMSO was added to reach 0.1% in the final incubation solutions. Concentrations ranged from 0.001 to 100 nM in case of ZEN and its metabolites and 0.001 to 100 µM in case of ISF, with 1:10 dilution steps in between. E2 (1 nM) served as positive control and 4-OH-TAM (10 µM) as a negative control. All experiments were performed in technical triplicates and at least five independent biological replicates.

Following the 24-h incubation, the supernatants were discarded and 100 µL of a ONE-Glo™ EX luciferase incubation solution (1:1 dilution of OGL reagent and EMEM (5% CD-FBS)) was added and shaken for 5 min. Subsequently, 90 µL of the supernatant of each well was transferred to a new, white 96-well plate. The luminescence of the white plate was directly measured with a gain of 140 using the Victor V3 1240 Multilabel Counter from Perkin Elmer (Waltham/MA, USA) or the Cytation 3 Cell Imaging Multi-Mode Reader from Biotek® (Winooski/VT, USA). Final results were referred to the solvent control (0.1% in DMSO) in percentage.

## OGL assay stability approach

Cell seeding was performed as described in the "ONE-Glo™ EX luciferase (OGL) assay" section. Subsequently, cells were incubated with 10 nM ZEN for 24 h. Following the incubation, the supernatant was removed and 100 µL of the OGL incubation solution was added and shaken for 5 min. Subsequently, 90 µL of the supernatant of each well was transferred to a new, white 96-well plate and right before measuring the bioluminescence, 10 µL of different concentrations (0.01–100 µM) of the ISF (GEN, DAI, EQ) were added to investigate whether the signal intensity is increasing over a 3-h period. The luminescence of the white plate was measured with a gain of 140 as stated above.

## Coupled CellTiter-Blue® and SRB cytotoxicity assay

The incubation solutions were prepared as described for the OGL assay (see respective section) and the same single substances, combinations thereof and solvent control (0.1%) were used. After the 3-h seeding period of the cells, the plates were incubated with the compound of interest for 24 h. All experiments were performed at least in five independent biological replicates with technical triplicates each. Following the incubation period, the subsequent steps were carried out in the dark: The supernatant was aspirated and 100 µL of a 1:10 CellTiter-Blue® (CTB) and EMEM (5% CD-FBS) solution were added and incubated for 50 min. Thereafter, 80 µL of each well was transferred into a black microwell plate and fluorescence was measured using an excitation wavelength of 560 nm and an emission wavelength of 590 nm with a gain of 65 with the Victor V3 1240 Multilabel Counter from Perkin Elmer (Waltham/MA, USA) or the Cytation 3 Cell Imaging Multi-Mode Reader from Biotek® (Winooski/VT, USA). Final results were referred to the solvent control (0.1% DMSO) in percentage.

The SRB assay was conducted immediately after the CTB assay. Following the removal of the remaining CTB solution the cells in each well were fixed with 10 µL of a 50% trichloroacetic acid solution in distilled water. The 96-well plates were placed for 1 h into the refrigerator at 4 °C. Upon this cold incubation, the plates were carefully rinsed twice with tap water and then left to dry in a dark surrounding. Once the plates had dried, 50 µL of the SRB solution was pipetted into each well. The dye was left to stain the proteins for 1 h, again in a dark setting at room

temperature. Afterwards the dye was rinsed off twice with tap water and twice with a 1% acetic acid solution. Here great care was taken to avoid the removal of the stained proteins from the bottom of the wells and the well plates were left to dry in the dark. Once the plates had dried, the SRB solution bound to the protein components of the cells was dissolved in 100 µL of an alkaline Tris base solution (0.30 g tris(hydroxymethyl-) aminomethane solved in 250 mL distilled water) by shaking for 5 min in the plate reader (Victor V3 1240 Multilabel Counter from Perkin Elmer (Waltham/MA, USA) or the Cytation 3 Cell Imaging Multi-Mode Reader from Biotek® (Winooski/VT Vermont, USA)). Subsequently, the absorbance was measured at 570 nm and as for the CTB; the final results were referred to the solvent control (0.1% DMSO) in percentage.

### Quantitative real-time PCR (RT-qPCR)

For the RT-qPCR, cells (hERα-HeLa-9903) were seeded in assay medium in 12-well plates at a density of 150,000 cells and allowed to grow for 24 h. Incubation solutions contained 0.1% DMSO and the test compounds and compound mixtures at various concentrations. After the same incubation time as the OGL-assay (24 h), cells were washed and subsequently the total RNA was extracted following the RNeasy Mini Kit protocol from Qiagen. The following reverse transcription was performed according to the QuantiTect-Reverse-Transcription manual (Qiagen 2009) to transcribe 1 µg RNA to cDNA. Then, RT-qPCR was performed using primers for the firefly luciferase encoding gene with the following oligonucleotide sequences for reverse primer 5′-GCCTCACCTACCTCC TTGCT-3′ and forward primer 5′-CTTCGTGACTTC CCATTTGC-3′, as well as the endogenous control primers delta-aminolevulinate synthase (1HS_ALAS1_1_SG, QT00073122), actin beta (Hs_ACTB_1_SG, QT00095431) and glyceraldehyde-3-phosphate dehydrogenase (Hs_GAPDH_1_SG, QT00079247) with SYBR green as fluorescent probe. The amplification the following setting was applied: 95 °C for 2 min, followed by 40 repetition cycles of: 95 °C (15 s), 55 °C (15 s), 72 °C (60 s). Thereafter, a melting curve analysis using the following parameters was performed: 15 s at 95 °C, 1 min at 60 °C, in 0.5 °C steps to 94 °C for 15 s. All samples were normalized to the mean of the endogenous control genes, in the case of GEN and combinations thereof (β-actin and GAPDH) and for DAI and EQ and mixtures thereof (ALAS1 and GAPDH). Quantification was performed according to Livak and Schmittgen (2001) using the 2-ΔΔCt method resulting in the depiction of fold-changes in comparison to the solvent control. We tested the transcriptional activity of GEN, DAI and EQ (0.1, 1 µM), and ZEN (10 nM) and combinations of ISF (1 µM) and ZEN (10 nM).

### Statistics

In order to generate substantial data, the measurements of OGL assay and cytotoxicity concerning the various combinations of ISF, ZEN, and ZEN metabolites were conducted in technical triplicates in addition to a minimum of five independent biological replicates. RT-qPCR experiments were performed in technical duplicates and in at least four independent biological replicates ($n \geq 4$). Then, the mean values for the biological replicates were calculated upon eliminating outliers based on the method of Nalimov and verifying normality following Shapiro-Wilk.

Further statistical analysis was performed using the software Origin Pro® 2021 (OriginLab Corporation, Northampton/MA, USA), with significance levels of 5%, 1%, and 0.1%, respectively (#, x = $p < 0.05$; ##, xx = $p < 0.01$; ###, xxx = $p < 0.001$). Significant differences were evaluated via one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test. Cytotoxicity and RT-qPCR results were evaluated by using one-way Student's *t*-test.

## Results

### OGL assay single substances

The induction of an estrogenic response for both mycoestrogens ZEN and α-ZEL was approximately the same. A concentration dependent increase in signal was observed and a concentration of 100 nM showed the highest luciferase induction of 95% (α-ZEL) and 86% (ZEN), related to 1 nM E2 (Fig. 2). Furthermore, in a concentration dependent manner the tested ISF induced an increase in luciferase activity up to a concentration of 10 µM with the highest induction by GEN, followed by DAI and EQ, with 345%, 342%, and 334%, respectively (Fig. 2). At a concentration of 100 µM, a decrease in luciferase induction was observed for all three ISF.

Exemplarily for combinations of DAI, additional assays with the ER antagonist 4-OH-TAM (10 µM) were conducted, according to the OECD protocol 455 for unambiguous confirmation of ER-mediated estrogenic activity (OECD 2015). 4-OH-TAM inhibits the activity of the ERs and by using it as an antagonist; it can be assessed whether the observed effects of a test substance are indeed mediated by the estrogen receptor. If the effects are reduced in the presence of 4-OH-TAM, it suggests that the test substance is likely interacting with the estrogen receptor. This helps in differentiating between estrogenic effects mediated through the estrogen

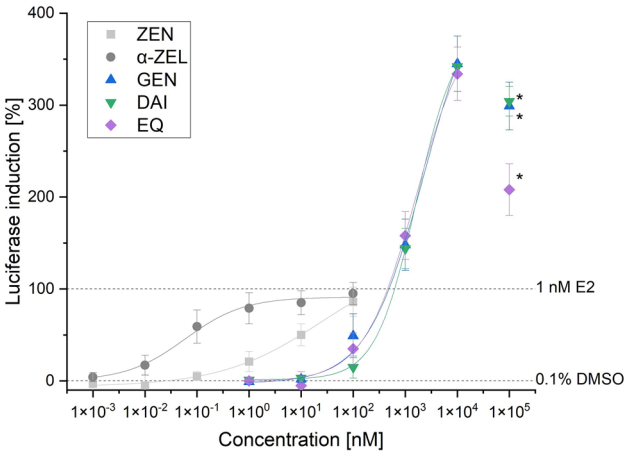


**Fig. 2** Sigmoidal-dose-response curve fits (created with: Origin) of the estrogen-dependent activation of luciferase induction in hERα HeLa 9903 cells caused by ZEN, α-ZEL and the ISF GEN, DAI and EQ after 24-h incubation. Results are depicted as mean ± standard deviation of at least 7 biological replicates (measurements with different cell passages), calculated from the mean value of three technical replicates (repeated measurements with the same cell passage on the same plate). Outliers after the Nalimov outlier test as well as values marked with asterisk (*) were not included in the sigmoidal-dose-response curve fit. Effects of the solvent control (0.1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively

receptor and other non-specific effects that might occur due to different mechanisms. 4-OH-TAM significantly lowered these apparent estrogenic activities, although at higher concentrations of ISF (≥ 1 µM), nonspecific interactions were also observed (Fig. 3). The true estrogenic activity that is mediated through ERα is calculated as the differences between 4-OH-TAM untreated and treated samples. Nevertheless, the higher transcriptional activity of DAI (≥ 1 µM) compared to E2 remains even after calculating the difference between 4-OH-TAM treated and untreated samples.

By calculating sigmoidal dose-response curve fitting the effective concentration that induces 50% response ($EC_{50}$) can be determined. The obtained $EC_{50}$ values for the mycoestrogens were 0.057 ± 0.004 nM (α-ZEL) and 24.4 ± 0.4 nM (ZEN), and for the ISF 2.3 µM ± 0.4 (GEN), 1.6 ± 0.2 µM (EQ), and 1.6 ± 0.7 µM (DAI).

## OGL assay of combinations

All measured effects of single substances and combinations are compiled in the heatmaps (Figs. 4 and 5). Results are expressed as percentage of induction, where 0 and 100% represent the values of the solvent control (0.1% DMSO) and 1 nM E2, respectively. Significant luciferase activation of combinations compared to their respective single substances are indicated by "x" in the case of ISF and by "#" for mycoestrogens. The color code of these heatmaps indicates the strength of the effect, which enables a visual interpretation of results of all tested combinations.

For all data sets, binary mixtures of ISF with ZEN and α-ZEL showed similar trends. Overall, no significant increase of luciferase activity was observed for all combinations and concentrations compared to their respective single

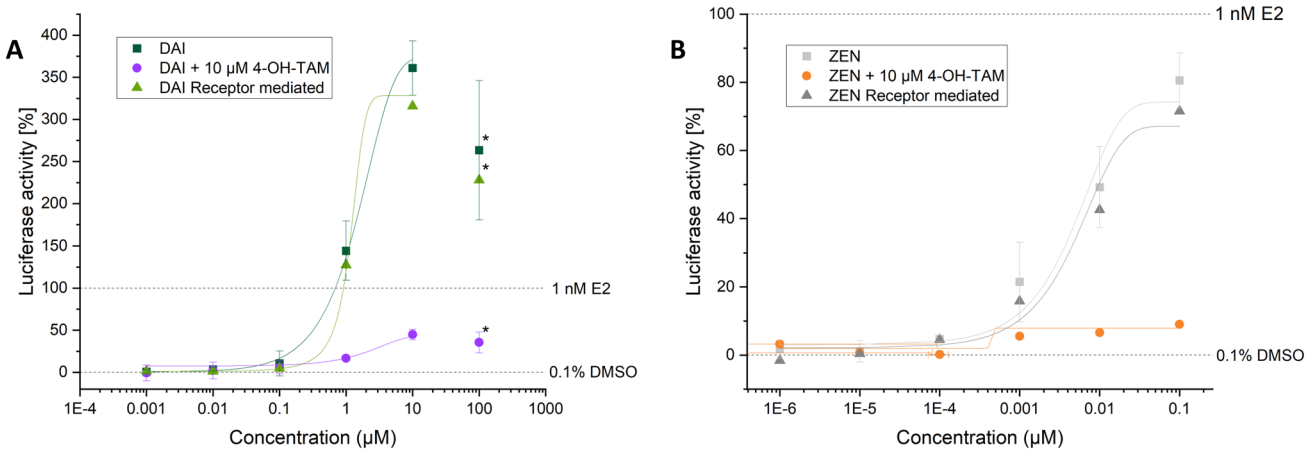


**Fig. 3** Total and receptor mediated (ERα) dose-response curves of DAI and ZEN (created with: Origin). **A** The dose-response curves of DAI in a concentration range between 0.001 µM and 100 µM are shown. In dark green the total luciferase activity induced by DAI is depicted while the red dots show the luciferase activity of DAI + 10 µM 4-OH-TAM (estrogen receptor α antagonist). In light green the calculated (total luciferase activity − the activity induced by DAI + 10 µM 4-OH-TAM) receptor mediated luciferase activity is shown. **B** The dose-response curves of ZEN in a concentration range between $1E^{-6}$ and 0.1 µM (hence a factor 1000 lower as tested for DAI). In light gray the total luciferase activity induced by ZEN is depicted. The orange dots show the luciferase activity of ZEN + 10 µM 4-OH-TAM and in dark gray the calculated (total luciferase activity − the activity induced by ZEN + 10 µM 4-OH-TAM) receptor mediated luciferase activity is provided. Outliers after the Nalimov outlier test as well as values marked with asterisk (*) were not included in the sigmoidal-dose-response curve fit. Effects of the solvent control (0.1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively

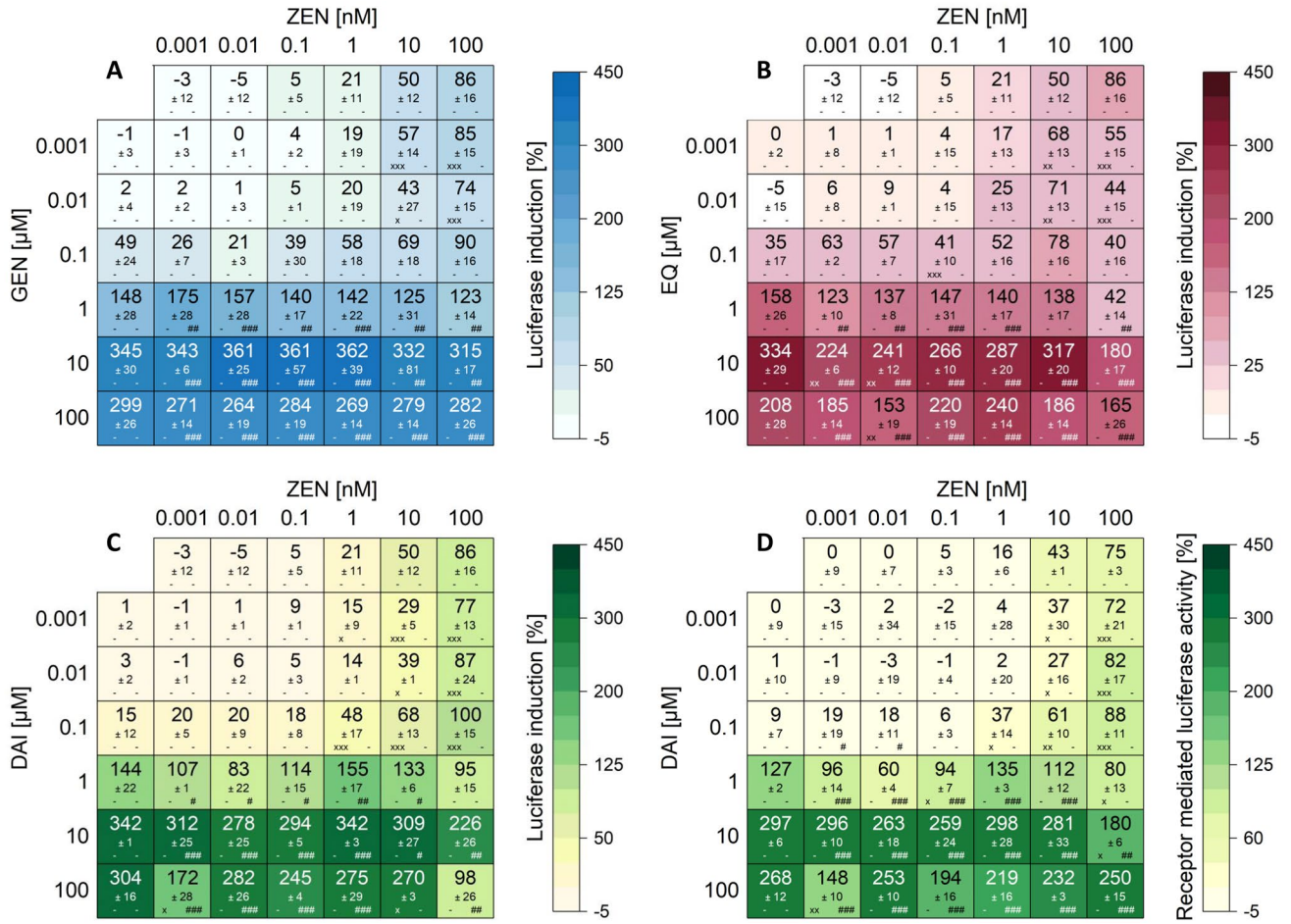


**Fig. 4** Effects of the combination of ISF with ZEN on the luciferase induction after 24 h of incubation using the hERα HeLa 9903 cell line (created with: Origin). Heatmaps indicating effects of single substances and combinations of GEN (**A**), EQ (**B**), DAI (**C**) and receptor mediated luciferase induction of DAI (**D**) with ZEN on the luciferase induction in hERα HeLa 9903 cells after 24 h incubation. Results are depicted as mean ± standard deviation of at least five biological replicates. Outliers after Nalimov outlier test were excluded. Effects of the solvent control (0.1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively. The color code indicates the strength of the effects. Normal distribution of data was tested according to Shapiro-Wilk normality test and significance by one-way ANOVA. Significant differences of effects to the respective single substance concentration were indicated with x = $p < 0.05$, xx = $p < 0.01$ and xxx = $p < 0.001$ in case of isoflavones and # = $p < 0.05$, ## = $p < 0.01$ and ### = $p < 0.001$ in case of ZEN. "-" corresponds to no significant difference to the respective concentration of the single substance

substances (Figs. 4 and 5). Only a few exceptions were able to potentiate the luciferase activity significantly (100 µM EQ + different α-ZEL concentrations) (Fig. 5B). ISF concentrations ≥ 1 µM alone and in combination with mycoestrogens surpassed the luciferase activation of 1 nM E2 (values above 100%) (Figs. 4 and 5). However, binary mixtures suppressed the luciferase activity compared to the respective single substances. Starting at a concentration of 0.001 µM for ISF, the addition of ZEN or α-ZEL (0.001–100 nM) lowered the induction of luciferase activity. This effect was more pronounced at higher ISF concentrations (1–100 µM). A concentration dependent increase in luciferase activity was observed for both mycoestrogens, whereas for all tested ISF, a decrease in luciferase activity was seen at the highest concentration of 100 µM. Furthermore, in combinations of 100 µM ISF together with ZEN or α-ZEL (0.001–100 nM) a decrease in luciferase activity was noticed.

## OGL assay stability approach

In the OGL stability approach, cells were incubated with 10 nM ZEN for 24 h and thereafter for 5 min with the OGL incubation solution. Thereafter, different ISF concentrations were added to the cell free supernatant. As seen in Fig. 6, ISF increased the stability of the enzyme which led to higher activities over time compared to the luciferase activity induced by ZEN alone. This was observed immediately after adding the ISF and over a time period of 3 h.

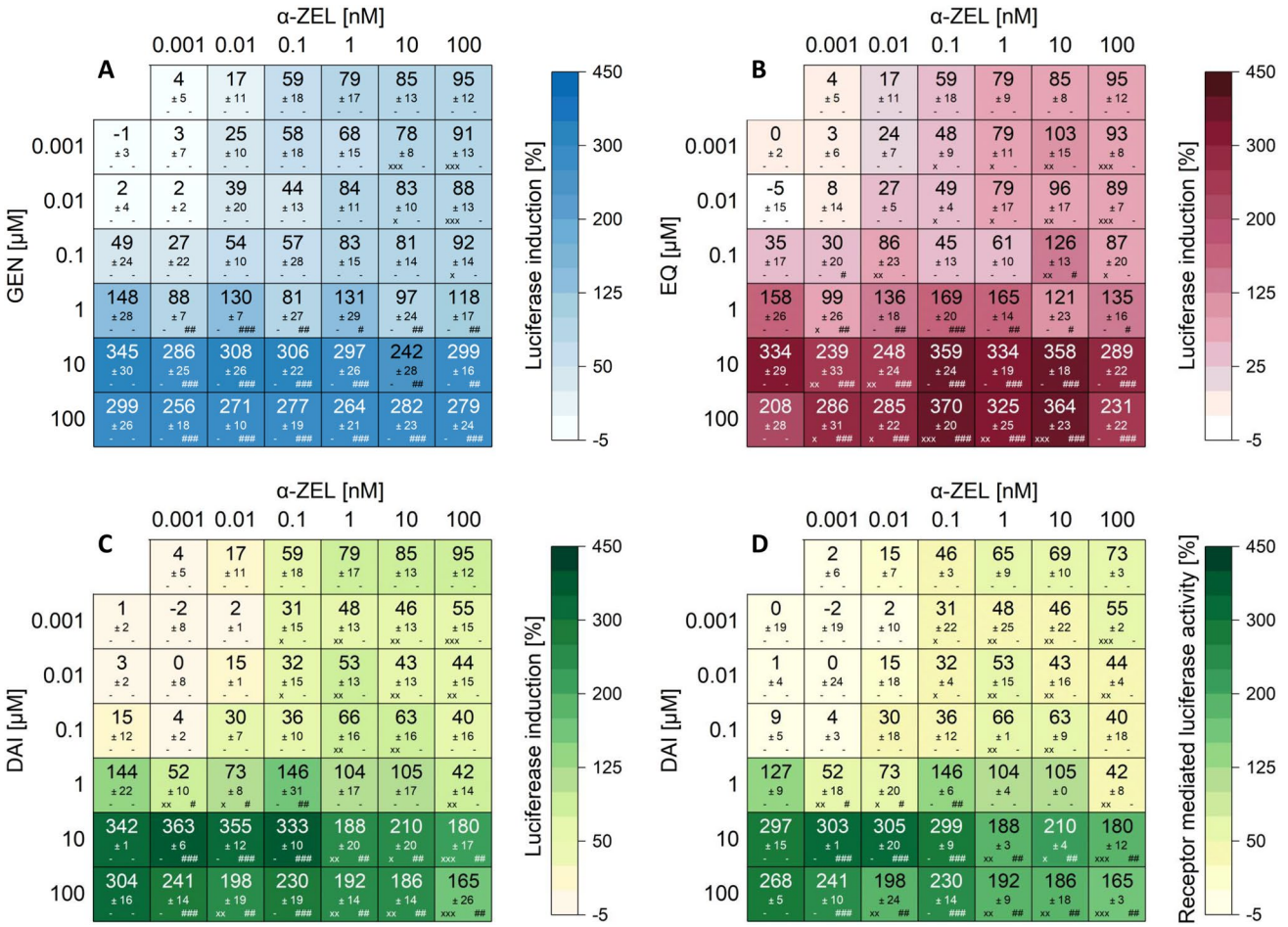


**Fig. 5** Effects of the combination of ISF with α-ZEL on the luciferase induction after 24 h of incubation using the hERα HeLa 9903 cell line (created with: Origin). Heatmaps indicating effects of single substances and combinations of GEN (**A**), EQ (**B**), and DAI (**C**) and receptor mediated luciferase induction of DAI (**D**) with α-ZEL on the luciferase induction in hERα HeLa 9903 cells after 24-h incubation. Results are depicted as mean ± standard deviation of at least five biological replicates. Outliers after Nalimov outlier test were excluded. Effects of the solvent control (0.1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively. The color code indicates the strength of the effects. Normal distribution of data was tested according to Shapiro-Wilk normality test and significance by one-way ANOVA. Significant differences of effects to the respective single substance concentration were indicated with $x = p < 0.05$, $xx = p < 0.01$, and $xxx = p < 0.001$ in case of the isoflavones and $\# = p < 0.05$, $\#\# = p < 0.01$, and $\#\#\# = p < 0.001$ in case of α-ZEL. The symbol "-" corresponds to no significant difference to the respective concentration of the single substance

## Cytotoxicity (CTB and SRB)

The metabolic activity and the protein content were assessed using the CTB and SRB assay, respectively, to determine the cytotoxicity of single compounds and combinations of phyto- and mycoestrogens. The purpose of the inclusion of cytotoxic measurements was to rule out possible artifacts induced by the compounds due to cell death or proliferation.

### Single substances

As single substances neither ZEN nor α-ZEL at all applied concentrations induced a reduction in metabolic activity in the utilized test system (Fig. 7A). Furthermore, neither the cell protein amount (SRB test system) was affected by these mycoestrogens in the same concentration range (Supplements Fig. 1A).

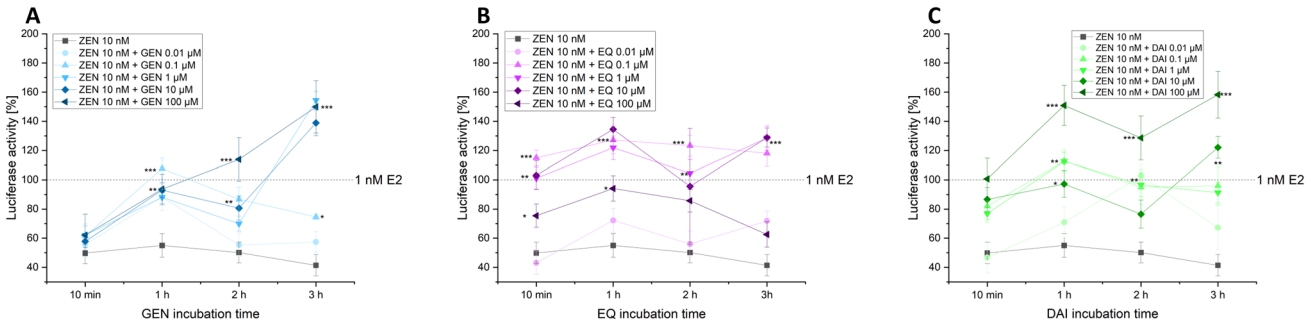


**Fig. 6** Effects of ISF on luciferase stability: Luciferase activity of ZEN alone and with different concentrations of GEN (**A**), EQ (**B**), and DAI (**C**) in hERα HeLa 9903 cells. The cells were incubated for 24 h with ZEN and the Isoflavones were added right before measuring the bioluminescence over a period of 3 h. Results are depicted as mean ± standard deviation of at least five biological replicates. Outliers after Nalimov outlier test were excluded. Effects of the solvent control (0.1% DMSO) and 1 nM E2 as positive control were set to 0 and 100%, respectively. Significant differences of effects to the respective single substance concentration were indicated with $*p<0.05$, $**p<0.01$, and $***p<0.001$. Please notice that the luciferase activity is only displayed between 40 and 200%. (created with: Origin)

For all three tested ISF no significant difference in metabolic activity was detected (Fig. 7B). Additionally, the protein content was not affected when compared to the solvent control (0.1% DMSO) (Supplements Fig. 1B).

### Cytotoxicity of combinations

As shown in Figs. 8 and 9, no significant decrease in metabolic activity for most of the tested combinations of binary mixtures of the tested phyto- and mycoestrogens was detected. However, for some combinations an increased tendency in the two applied systems was observed (Fig. 9B). Furthermore, the combinations of DAI with 10 µM 4-OH-TAM showed a decrease in number of cells compared to the cells which were untreated with 10 µM 4-OH-TAM, albeit just a few significant differences were detected (Fig. 9D). Moreover, the cell protein amount using the SRB assay, showed for most combinations no significant differences compared to the solvent control. Cells treated with 10 µM 4-OH-TAM showed a tendency to decrease the protein content; however, for most combinations, this trend was not significant.

## RT-qPCR

In the applied concentrations, ISF only increased the transcriptional activity slightly and were not able to surpass the induction of luciferase mRNA induced by 1 nM E2

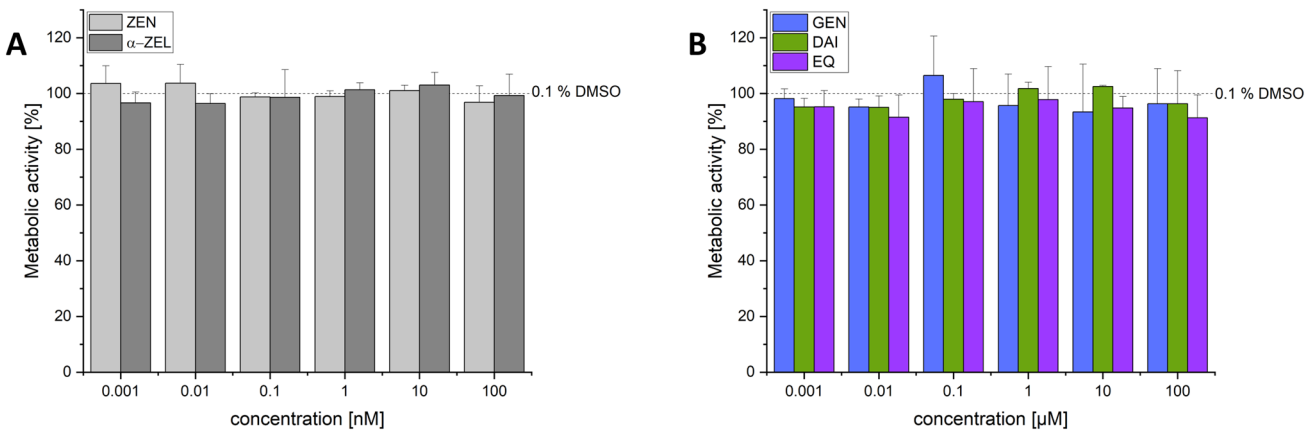


**Fig. 7** Effects of the single substances on the metabolic activity (%) measured by CTB after 24-h incubation in hERα HeLa 9903 cells for ZEN and α-ZEL (**A**) and GEN, DAI and EQ (**B**). Values were referred to the solvent control (0.1% DMSO) as 100%. Results are depicted as mean + standard deviation of at least eight biological replicates, calculated from the mean value of three technical replicates. Outliers after Nalimov outlier test were excluded. Significant differences of effects between the solvent control and the incubation solutions were calculated by one-sample Student's t-test ($p<0.05$), but no significant difference was observed. (created with: Origin)

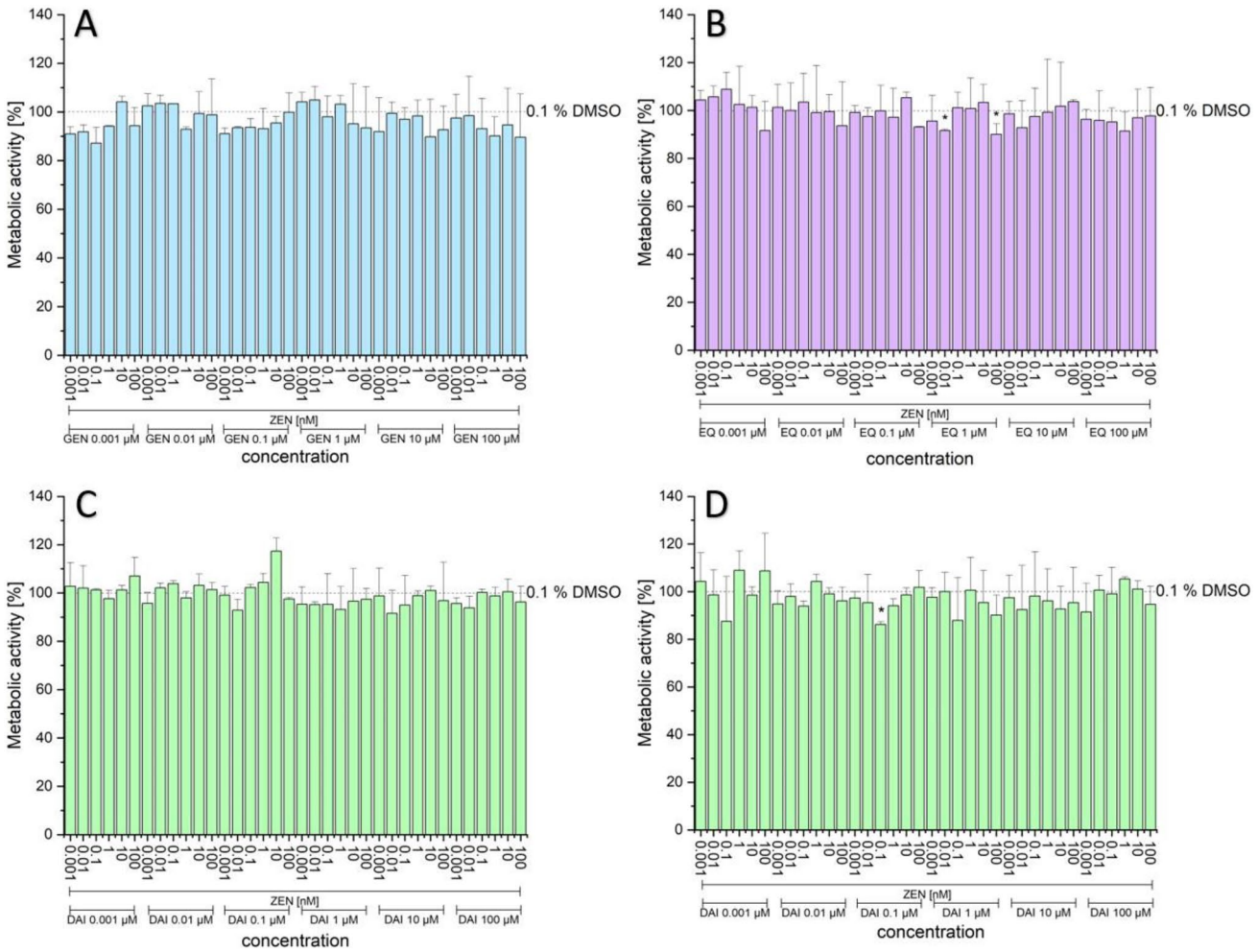


**Fig. 8** Effects of the combination of ZEN with ISF on the metabolic activity (%) measured by CTB of different combinations and concentrations after 24-h incubation in hERα HeLa 9903 cells. GEN (**A**), EQ (**B**), DAI (**C**), and DAI + 10 µM 4-OH-TAM (**D**) with ZEN (nM). Values were referred to the solvent control (0.1% DMSO) as 100%. Results are depicted as mean + standard deviation of at least four biological replicates, calculated from the mean value of three technical replicates. Outliers after Nalimov outlier test were excluded. Significant differences of effects between the solvent control and the incubation solutions were calculated by one-sample Student's t-test. Significances are indicated with asterisk (*) ($p < 0.05$). (created with: Origin)

(Fig. 10). ZEN induced luciferase mRNA levels significantly and was comparable to the induction of luciferase using the OGL assay. The combination of 1 µM ISF and 10 nM ZEN showed the same increase in luciferase mRNA levels as ZEN alone. For the RT-qPCR experiments with GEN, β-actin and GAPDH were used as housekeeping genes, whereas for DAI and EQ, GAPDH, and ALAS1 were applied. As a result, different fold changes of the luciferase encoding gene were observed when comparing the experiments.

## Discussion

As previously reported, it was demonstrated that binary mixtures of ISF together with ZEN and several of its metabolites have synergistic estrogenic effects using the Ishikawa cell model, which expresses both isoforms of the ER (Grgic et al. 2022). It is hypothesized that these enhanced estrogenic effects are based on the presence of both ER isoforms α and β. While ZEN and its metabolites have a relative high

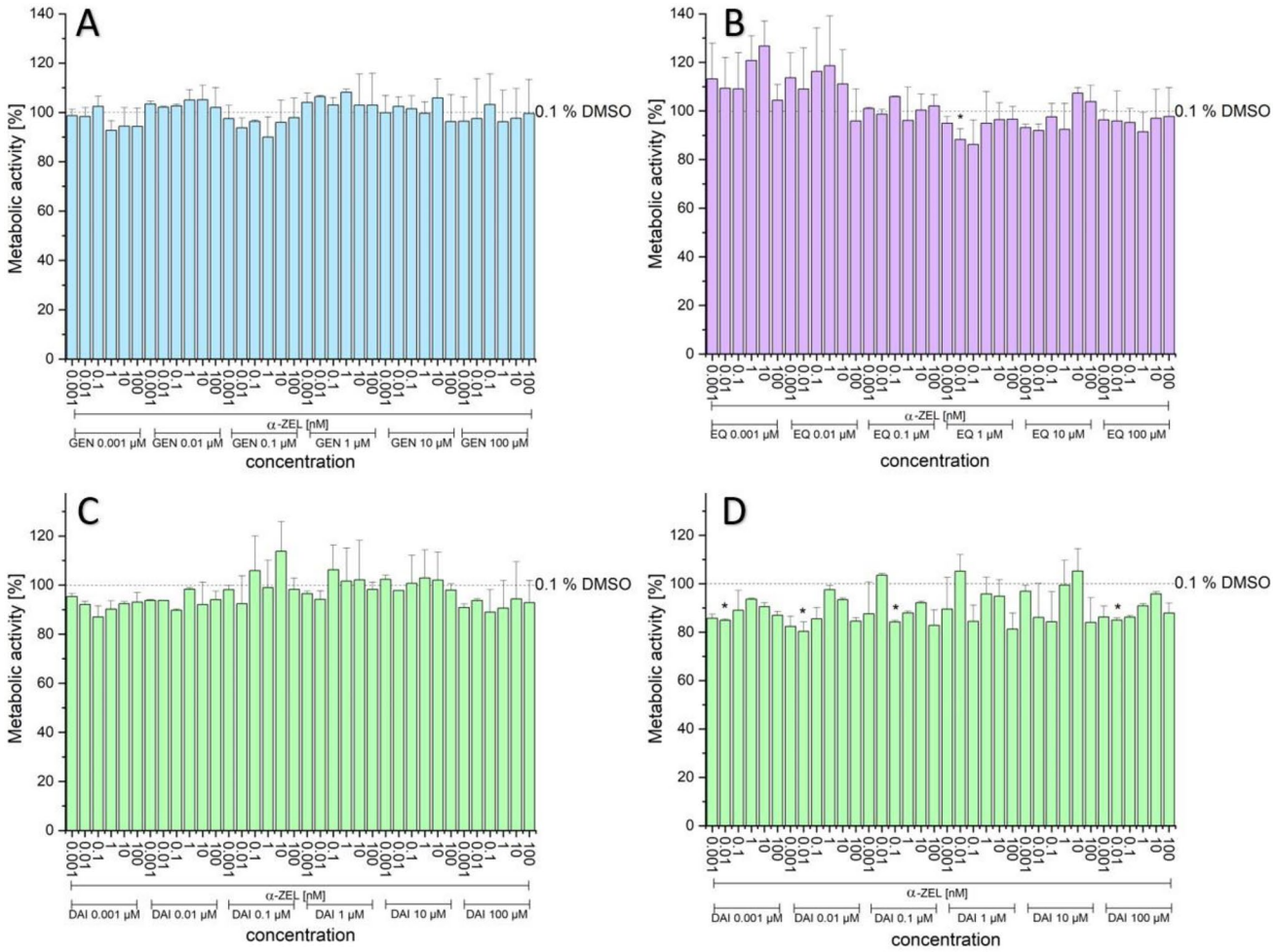


**Fig. 9** Effects of the combination of α-ZEL with ISF on the metabolic activity (%) measured by CTB of different combinations and concentrations after 24-h incubation in hERα HeLa 9903 cells. GEN (**A**), EQ (**B**), DAI (**C**), and DAI + 10 µM 4-OH-TAM (**D**) with α-ZEL (nM). Values were referred to the solvent control (0.1% DMSO) as 100%. Results are depicted as mean + standard deviation of at least four biological replicates, calculated from the mean value of three technical replicates. Outliers after Nalimov outlier test were excluded. Significant differences of effects between the solvent control and the incubation solutions were calculated by one-sample Student's t-test. Significances are indicated with asterisk (*) ($p < 0.05$). (created with: Origin)

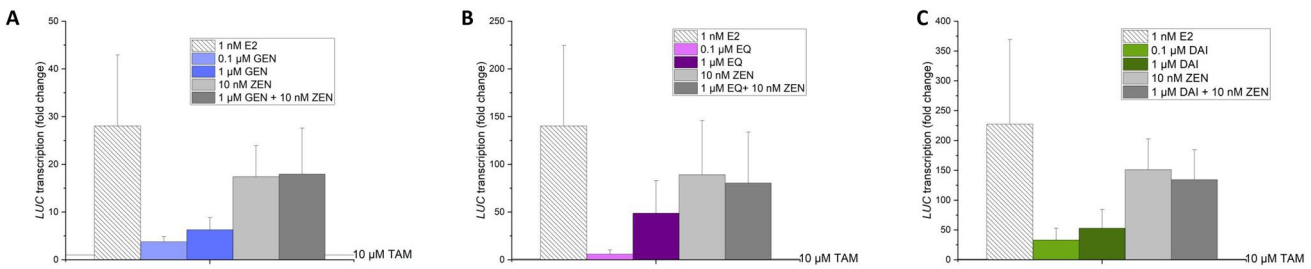


**Fig. 10** Effects of ISF and ZEN and combinations thereof on gene transcription (created with: Origin). Alterations in gene transcription of hERα HeLa 9903 cells incubated for 24 h with 1 nM E2, 10 µM 4-OH-TAM, 0.1 and 1 µM of GEN, EQ and DAI, 10 nM ZEN and combinations of 1 µM GEN, EQ and DAI with 10 nM ZEN. Results are depicted as mean ± standard deviation of at least four biological replicates. Outliers after Nalimov outlier test were excluded. For the RT-qPCR experiments with GEN, β-actin and GAPDH were used as housekeeping genes, whereas for DAI and EQ, GAPDH and ALAS1 were applied. As a result, different fold changes of the luciferase encoding gene were observed when comparing the experiments

affinity to bind to both ERs, ISF preferably interact with ERβ (Nikov et al. 2000; Setchell et al. 2005; Takemura et al. 2007). In the present study, we tested selected combinations using the hERα-HeLa-9903 cell model expressing solely the ERα. According to an OECD protocol, this assay should be used for detecting estrogen receptor agonists and antagonists [13]. Based on the previous results our hypothesis was that no enhanced estrogenic effects should be observed in the hERα-HeLa-9903 cell line due to the lack of ERβ.

Performing the OGL assay, α-ZEL in a concentration range between 0.01 and 10 nM had a higher potency to induce luciferase compared to ZEN. It is well known that the estrogenic effect of the phase I metabolite is more pronounced compared to its parent compound (Molina-Molina et al. 2014; Vejdovszky et al. 2017; Mendez-Catala et al. 2020; Grgic et al. 2022). At a concentration of 100 nM, both mycoestrogens had a similar impact on luciferase induction of 95% (α-ZEL) and 86% (ZEN). Interestingly, when comparing the estrogenic effect of these two mycoestrogens between different cell lines, 10 times higher concentrations were required in hERα-HeLa-9903 cells to induce the same estrogenic effects when compared to Ishikawa cells (Grgic et al. 2022). As for the ISF a superinduction of luciferase is observed, no direct comparison to other cell lines is possible. This superinduction of luciferase by ISF in hERα-HeLa-9903 cells was already described by Gramec Skledar et al. and was in accordance with the results of these experiments (Gramec Skledar et al. 2020).

When examining the luciferase response triggered by mycoestrogens and ISF, it was observed that ZEN and α-ZEL initiated an increase in luciferase activity at concentrations of 0.1 and 0.01 nM, respectively. In contrast, ISF demonstrated a noticeable signal increase starting at a concentration of 100 nM. This divergence in concentration thresholds suggests the higher binding affinity of ISF to the estrogen receptor alpha (ERα), underscoring the superior estrogenic potency of mycoestrogens compared to ISF. It is worth noting, however, that while ZEN and α-ZEL exhibited substantial luciferase induction, they did not surpass the induction levels achieved by E2, a known estrogenic compound. Conversely, ISF showed the ability to exceed the activities of E2. This, however, does not fully reflect receptor-mediated interactions and is known as the phenomena superinduction.

As expected, combinations of mycoestrogens and ISF did not show increased luciferase activity compared to the induction of their respective single substances (Figs. 4 and 5). However, ISF was found to induce luciferase activities up to three times higher compared to the positive control of 1 nM E2.

Therefore, the next step was to verify whether or not the increased firefly luciferase activity is a true reflection of increased transcriptional activity. Thus, the mRNA expression of the firefly luciferase gene was measured in ERα-HeLa-9903 cells. We tested the transcriptional activity of GEN, DAI and EQ (0.1, 1 µM), and ZEN (10 nM) and combinations of ISF (1 µM) and ZEN (10 nM) (Fig. 10). Those concentrations were chosen based on the results of the OGL assay aiming for a concentration without (0.1 µM) and with (1 µM) superinduction in luciferase. This allowed the assessment of the validity of the OGL-experiment for ISF. In the applied concentrations, ISF only slightly increased the transcriptional activity and were not able to surpass the induction of luciferase mRNA induced by 1 nM E2. This indicates that the superinduction observed by the ISF in the ERα-HeLa-9903 cells is not mediated by the receptor activation and therefore, not a true reflection of its binding affinity to the ERα in this system. ZEN was able to induce luciferase mRNA levels significantly and was comparable to the induction of luciferase in ERα-HeLa-9903 cells. The combination of 1 µM GEN and 10 nM ZEN showed the same increase in luciferase mRNA levels as ZEN alone. This observation would support the theory that for synergistic estrogenic effects both estrogen receptors are required, with different binding affinities of two substances to the ER α and β.

It is known that there might be a direct interaction of ISF with the reporter enzyme leading to stabilization (decreased degradation) of the enzyme. It has been proposed that certain compounds directly bind to, and stabilize, the firefly luciferase reporter enzyme thereby increasing its half-life (Pfitscher et al. 2008). The interaction between the bioactive chemicals and the luciferase results in inhibition of the enzyme activity at the same time resulting in stabilization of the enzyme (Thomas 2008). These luciferase-stabilizing compounds are referred to in the literature as luciferase inhibitors.

It can be expected that upon rupture of the cells the inhibitor will dissociate leading to increased activity of the luciferase enzyme due to this stabilizing effect. Thus, ISF at concentrations above 1 µM may interact with luciferase thereby stabilizing the enzyme so that upon lysis of the cells and dissociation of ISF from the enzyme due to dilution, increased activity can be measured as compared to the situation without an added stabilizer/inhibitor (Sotoca et al. 2010). It has been recently shown, that a series of bioactive compounds, that inhibit and thereby stabilize the firefly luciferase enzyme will result in an increased luminescence signal (Pfitscher et al. 2008).

Therefore, in a further approach, we performed a luciferase reporter assay to determine if the ISF are able to increase the stability and protect against degradation. We incubated the cells for 24 h with 10 nM ZEN. Subsequently, right before measuring the bioluminescence, different concentrations (0.01–100 µM) of ISF were added to investigate whether the signal intensity is increasing over a 3-h period.

As depicted in Fig. 6, the addition of different concentrations of ISF to 10 nM ZEN increased the stability of the enzyme which led to higher activities over time compared to the luciferase activity that was induced by ZEN alone. The addition of ISF independent of the applied concentration led to increased signal intensity. This was observed immediately after adding the ISF and increase over this relative short incubation time. This observation supports our hypothesis that ISF increase the stability of luciferase and therefore such high bioluminescence signals can be induced by ISF as seen in the experiments of combinations between ISF and ZEN.

To conclude, in our experiments we aimed to display the importance of the presence of both isoforms of the ER, ERα, and ERβ to induce synergistic estrogenic effects upon co-occurrence of myco- and phytoestrogens. Indeed, in cell lines bearing only one isoform of the ER, as it was the case for the hERα HeLa 9903, no enhancement in estrogenicity was observed up to a concentration of 0.1 µM of the ISFs. In higher ISF concentrations, we elucidated that assays according to the OECD guideline 455 are not a true reflection of receptor activation and are therefore not applicable for interaction studies with these types of compounds. When applying the OGL assay, it is important to test for any inferences resulting in the non-applicability of the test system.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s12550-023-00506-1.

**Funding** Open access funding provided by University of Veterinary Medicine Vienna This research was funded by the Austrian Research Promotion Agency (FFG) and BIOMIN Holding GmbH through the Bridge project "ISOMYCOTOX – Combinatory endocrine activity of mycoestrogens and soy isoflavones in porcine feed" (project number 880656).

## Declarations

**Conflict of interest** The authors declare no competing interests.



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# 9. Abstract & Zusammenfassung

## 9.1 Abstract

Soy has long been a mainstay as a protein source in animal feed, driven by its nutritional attributes. It has been associated with various benefits, including growth promotion, bolstered antioxidative capacities, and enhanced immune functions due to the presence of isoflavones (ISF). However, this landscape is not devoid of complexities. Research suggests that elevated ISF intake, while beneficial in many aspects, can have detrimental consequences, particularly concerning the reproductive health of female farm animals, potentially leading to issues such as infertility, uterine prolapse, and glandular abnormalities.

Of intriguing note is the frequent co-presence of isoflavones and the mycotoxin zearalenone (ZEN) in animal feeds. ZEN, generated by certain *Fusarium* species, is a secondary fungal metabolite commonly detected in grains and legumes. ZEN is notorious for its varied toxic effects on both humans and animals, with a central facet being its potent endocrine-disrupting prowess. Its metabolite α-zearalenol (α-ZEL) demonstrates even stronger estrogenic effects, while certain phase II metabolites, like ZEN-14-glucoside, exhibit a loss of estrogenic properties.

Both ISF and ZEN share structural and functional similarities with the natural hormone 17-β-estradiol (E2), rendering them xenoestrogens that activate estrogen receptors (ER), although their affinities toward different ER isoforms α and β vary. Notably, ZEN has a greater affinity for ERα, whereas ISF exhibit heightened affinity for ERβ. This distinction hints at the possibility of augmented estrogenic effects when these two groups of compounds coexist.

Considering these aspects, regulatory thresholds for ZEN in food and feed have been established, accounting for its potential adverse health effects. However, current risk assessments primarily focus on the individual toxicological profiles of single compounds, potentially underestimating the cumulative toxicity of mixtures. Consequently, a comprehensive evaluation of compound interactions is needed for more robust safety evaluations.

In this thesis the effects of various substances on estrogenic activity, particularly focusing on mycotoxins and phytoestrogens were studied. The research revealed that alpha-zearalenol (α-ZEL) is the most potent estrogen, followed by alpha-zearalanol (α-ZAL) and ZEN, in the Ishikawa cell line. On the other hand, ZEN-

14-sulfate (ZEN-14-S) showed no estrogenic potential due to its conjugated form, as observed through experiments and previous computational studies.

While ZEN can induce cytotoxic effects at very high concentrations, the concentrations used for the studies in the Ishikawa cells (ranging from 0.001 to 10 nM) did not lead to significant cytotoxicity. Interestingly, some concentrations of ZEN and its metabolites actually triggered an increase in cellular activity, which might be due to mitochondrial swelling or other mechanisms. This suggests that these mycotoxins could have proliferative effects.

Phytoestrogens, found in soy-based products, were tested at various concentrations to assess their estrogenic potency. The results showed that genistein (GEN), daidzein (DAI), and equol (EQ) increased the expression of ALP (a marker of estrogenic activity) in a concentration-dependent manner. However, GLY only had a limited impact on ALP expression, even at higher concentrations. In comparison to ZEN and its derivatives, much higher concentrations of phytoestrogens were needed to achieve similar estrogenic effects.

Further investigations into the interactions between mycotoxins and phytoestrogens revealed that combinations of these substances often resulted in higher ALP induction compared to individual compounds. The conducted experiments also suggested synergistic estrogenic effects between certain mycotoxins and phytoestrogens, particularly at lower concentrations. However, as ISF concentrations increased, the interactions tended to become additive or even antagonistic. The combination of ZEN and EQ exhibited the most potent estrogenic effect.

It is hypothesized that the interactions between phytoestrogens and mycotoxins were influenced by their interactions with estrogen receptors (ERs), specifically ERα and ERβ. While ZEN and its metabolites had stronger affinities for both ERs, phytoestrogens primarily interacted with ERβ. This interaction pattern likely contributed to the observed synergistic effects.

In conclusion, the study highlighted the complex interactions between mycotoxins and phytoestrogens, indicating that their combined effects need to be considered for risk assessment. The potential for enhanced estrogenic effects, especially in the presence of certain combinations, suggests a need for reevaluating safety limits and understanding the implications, particularly for livestock feed and human consumption. Further research and *in vivo* studies are essential to confirm these findings and their broader implications for consumer safety.

In the experiments conducted in Ishikawa, we demonstrated that combinations of certain ISF with ZEN and its metabolites have synergistic estrogenic effects, particularly in cell lines expressing both ER isoforms (ERα and ERβ). We proposed that these enhanced effects arise from the presence of both ER isoforms. Tested these combinations in cell lines expressing only ERα, as per OECD guidelines, we did not observe enhanced estrogenic effects due to the lack of ERβ.

Using a luciferase reporter assay, we found that α-ZEL had a higher potency to induce luciferase than ZEN at concentrations between 0.01 and 10 nM. It is known that phase I metabolites like α-ZEL often have stronger estrogenic effects than their parent compounds. At a concentration of 100 nM, both mycoestrogens had similar effects on luciferase induction. Interestingly, when comparing these compounds' estrogenic effects between different cell lines, we noted that higher concentrations were needed in cells expressing only ERα to achieve the same effects as in cells expressing both ER isoforms.

Combinations of mycoestrogens and ISF did not show increased luciferase activity compared to single substances. However, ISF induced luciferase activities up to three times higher than the positive control (E2), but this was not mediated by receptor activation. To confirm if increased firefly luciferase activity truly reflected increased transcriptional activity, we measured mRNA expression of the luciferase gene in cells expressing only ERα. ISF only slightly increased transcriptional activity and could not surpass the effect induced by E2, indicating the observed superinduction by ISF in these cells is not directly mediated by ERα.

We explored whether ISF interact with luciferase, stabilizing the enzyme and affecting its degradation. Indeed, when we incubated cells with ZEN and then added ISF, we observed increased stability of the enzyme, leading to higher activity over time. This supported our hypothesis that ISF can stabilize luciferase, explaining the high bioluminescence signals observed in combination experiments.

In summary, our findings emphasized the need for both ER isoforms for synergistic estrogenic effects and suggested that OECD guideline 455 might not be applicable for testing interactions involving ISF at concentrations above 1 µM. We highlighted the importance of understanding the test system's limitations when applying the luciferase assay and provided insights into the interaction between ISF and the luciferase enzyme.

## 9.2 Zusammenfassung

Soja ist schon lange eine Hauptquelle für Protein in Tierfutter, auch durch seine ernährungsphysiologischen Eigenschaften. Es wurde mit verschiedenen Vorteilen in Verbindung gebracht, darunter Wachstumsförderung, gestärkte antioxidative Kapazitäten und verbesserte Immunfunktionen aufgrund des Vorhandenseins von Isoflavonen (ISF). Allerdings ist dieser Bereich nicht frei von Komplexitäten. Forschungsergebnisse deuten darauf hin, dass eine erhöhte Aufnahme von ISF, obwohl in vielerlei Hinsicht vorteilhaft, nachteilige Folgen haben kann, insbesondere im Hinblick auf die reproduktive Gesundheit weiblicher Nutztiere, was potenziell zu Problemen wie Unfruchtbarkeit, Gebärmuttervorfall und Drüsenanomalien führen kann.

Besonders interessant ist die häufige gemeinsame Anwesenheit von Isoflavonen und dem Mykotoxin Zearalenon (ZEN) in Tierfuttermitteln. ZEN, das von bestimmten *Fusarium*-Arten erzeugt wird, ist ein sekundärer Pilzmetabolit, der in Getreide und Hülsenfrüchten häufig nachgewiesen wird. ZEN ist berüchtigt für seine vielfältigen toxischen Wirkungen sowohl auf Menschen als auch auf Tiere, wobei ein zentraler Aspekt seine starke endokrine Störungsfähigkeit ist. Sein Metabolit α-Zearalenol (α-ZEL) zeigt noch stärkere östrogene Effekte, während bestimmte Phase-II-Metaboliten wie ZEN-14-Glucosid einen Verlust östrogenartiger Eigenschaften aufweisen.

Sowohl ISF als auch ZEN weisen strukturelle und funktionelle Ähnlichkeiten mit dem natürlichen Hormon 17-β-Östradiol (E2) auf, was sie zu Xenoöstrogenen macht, die Östrogenrezeptoren (ER) aktivieren, obwohl ihre Affinitäten zu den verschiedenen ER-Isoformen α und β variieren. Bemerkenswert ist, dass ZEN eine größere Affinität zu ERα aufweist, während ISF eine erhöhte Affinität zu ERβ zeigen. Diese Unterscheidung deutet auf die Möglichkeit verstärkter östrogenartiger Effekte hin, wenn diese beiden Gruppen von Verbindungen gemeinsam auftreten.

Angesichts dieser Aspekte wurden regulatorische Schwellenwerte für ZEN in Lebensmitteln und Futtermitteln festgelegt, um seine potenziell nachteiligen gesundheitlichen Auswirkungen zu berücksichtigen. Allerdings konzentrieren sich aktuelle Risikobewertungen hauptsächlich auf die individuellen toxikologischen Profile einzelner Verbindungen, wodurch die kumulative Toxizität von Mischungen möglicherweise unterschätzt wird. Daher ist eine umfassende Bewertung von Verbindungsinteraktionen erforderlich, um robustere Sicherheitsbewertungen zu ermöglichen. Unsere Studien zielten darauf ab, eine eingehende Erkundung der Wechselwirkungen zwischen Mykotoxinen und Phytoöstrogenen durchzuführen.

In dieser Dissertation wurden die Auswirkungen verschiedener Substanzen auf die östrogene Aktivität untersucht, wobei der Schwerpunkt insbesondere auf Mykotoxinen und Phytoöstrogenen lag. Die Forschung ergab, dass α-ZEL die stärkste Östrogenwirkung hat, gefolgt von α-ZAL und ZEN, in der Ishikawa-Zelllinie. Auf der anderen Seite zeigte ZEN-14-S aufgrund seiner konjugierten Form kein östrogenes Potenzial, wie es in Experimenten und früheren Berechnungen beobachtet wurde.

Während ZEN bei sehr hohen Konzentrationen zytotoxische Effekte auslösen kann, führten die für die Studien in den Ishikawa-Zellen verwendeten Konzentrationen (im Bereich von 0,001 bis 10 nM) nicht zu signifikanter Zytotoxizität. Interessanterweise lösten einige Konzentrationen von ZEN und seinen Metaboliten tatsächlich eine Zunahme der zellulären Aktivität aus, was auf mitochondriale Schwellungen oder andere Mechanismen zurückzuführen sein könnte. Dies legt nahe, dass diese Mykotoxine proliferative Effekte haben könnten.

Phytoöstrogene, die in sojabasierten Produkten vorkommen, wurden in verschiedenen Konzentrationen getestet, um ihre östrogene Potenz zu beurteilen. Die Ergebnisse zeigten, dass GEN, DAI und EQ die Expression von ALP (einem Marker für östrogene Aktivität) in abhängiger Konzentration erhöhten. GLY hatte jedoch selbst bei höheren Konzentrationen nur einen begrenzten Einfluss auf die ALP-Expression. Im Vergleich zu ZEN und seinen Derivaten waren wesentlich höhere Konzentrationen von Phytoöstrogenen erforderlich, um ähnliche östrogene Effekte zu erzielen.

Weitere Untersuchungen der Wechselwirkungen zwischen Mykotoxinen und Phytoöstrogenen ergaben, dass Kombinationen dieser Substanzen häufig zu einer stärkeren Induktion von ALP führten als einzelne Verbindungen. Die durchgeführten Experimente deuteten auch auf synergistische östrogene Effekte zwischen bestimmten Mykotoxinen und Phytoöstrogenen hin, insbesondere bei niedrigeren Konzentrationen. Mit steigenden ISF-Konzentrationen neigten die Wechselwirkungen jedoch dazu, additiv oder sogar antagonistisch zu werden. Die Kombination von ZEN und EQ zeigte den stärksten östrogenen Effekt.

Es wird vermutet, dass die Wechselwirkungen zwischen Phytoöstrogenen und Mykotoxinen von ihren Interaktionen mit Östrogenrezeptoren (ER), insbesondere ERα und ERβ, beeinflusst wurden. Während ZEN und seine Metaboliten eine stärkere Affinität zu beiden ER aufwiesen, interagierten Phytoöstrogene hauptsächlich mit ERβ. Dieses Interaktionsmuster trug wahrscheinlich zu den beobachteten synergistischen Effekten bei.

Zusammenfassend betonte die Studie die komplexen Wechselwirkungen zwischen Mykotoxinen und Phytoöstrogenen und deutete darauf hin, dass ihre kombinierten Effekte bei der Risikobewertung berücksichtigt werden müssen. Das Potenzial für verstärkte östrogene Effekte, insbesondere in Anwesenheit bestimmter Kombinationen, legt die Notwendigkeit einer Neubewertung von Sicherheitsgrenzen nahe und erfordert ein Verständnis der Implikationen, besonders für Nutztierfutter und menschlichen Konsum. Weiterführende Forschung und In-vivo-Studien sind unerlässlich, um diese Ergebnisse und ihre breiteren Auswirkungen auf die Verbrauchersicherheit zu bestätigen.

In den in Ishikawa durchgeführten Experimenten konnte gezeigt werden, dass Kombinationen bestimmter ISF mit ZEN und seinen Metaboliten synergistische östrogene Effekte haben, insbesondere in Zelllinien, die beide ER-Isoformen (ERα und ERβ) exprimieren. Es wird vermutet, dass diese verstärkten Effekte aufgrund der Anwesenheit beider ER-Isoformen entstehen. Während ZEN und seine Metaboliten an beide ER-Isoformen binden können, interagieren ISF hauptsächlich mit ERβ. Wenn wir jedoch diese Kombinationen in Zelllinien testeten, die nur ERα gemäß den OECD-Richtlinien exprimierten, beobachteten wir keine verstärkten östrogenen Effekte aufgrund des Fehlens von ERβ.

Mit Hilfe eines Luciferase-Reporter-Assays konnte gezeigt werden, dass α-ZEL eine höhere Potenz zur Induktion von Luciferase hatte als ZEN in Konzentrationen zwischen 0,01 und 10 nM. Es ist bekannt, dass Phase-I-Metaboliten wie α-ZEL oft stärkere östrogene Effekte als ihre Ausgangsverbindungen aufweisen. Bei einer Konzentration von 100 nM hatten beide Mykoöstrogene ähnliche Effekte auf die Luciferase-Induktion. Interessanterweise war zu beobachten bei Vergleichen der östrogenen Effekte dieser Verbindungen zwischen verschiedenen Zelllinien, dass höhere Konzentrationen in Zellen benötigt wurden, die nur ERα exprimierten, um dieselben Effekte zu erzielen wie in Zellen, die beide ER-Isoformen exprimierten.

Kombinationen von Mykoöstrogenen und ISF zeigten im Vergleich zu Einzelsubstanzen keine erhöhte Luciferase-Aktivität. ISF induzierten jedoch Luciferase-Aktivitäten, die bis zu dreimal höher waren als die des positiven Kontrollwertes (E2). Dies war jedoch nicht durch Rezeptoraktivierung vermittelt. Um zu bestätigen, ob die erhöhte Luciferase-Aktivität tatsächlich eine erhöhte transkriptionale Aktivität widerspiegelte, maßen wir die mRNA-Expression des Luciferase-Gens in Zellen, die nur ERα exprimierten. ISF erhöhten die transkriptionale Aktivität nur geringfügig und konnten den durch E2 induzierten Effekt nicht übertreffen. Dies deutet darauf hin, dass die beobachtete Superinduktion durch ISF in diesen Zellen nicht direkt durch ERα vermittelt wird.

Es wurde untersucht, ob ISF mit Luciferase interagieren und das Enzym stabilisieren und den Abbau beeinflussen. Tatsächlich beobachteten wir, dass die Stabilität des Enzyms erhöht wurde, wenn wir Zellen mit ZEN inkubierten und dann ISF hinzufügten, was im Laufe der Zeit zu einer höheren Aktivität führte. Dies unterstützte unsere Hypothese, dass ISF Luciferase stabilisieren können und erklärte die hohen Biolumineszenzsignale, die in Kombinationsversuchen beobachtet wurden.

Zusammenfassend betonten die Ergebnisse der vorliegenden Arbeit die Notwendigkeit beider ER-Isoformen für synergistische östrogene Effekte und legten nahe, dass die OECD-Richtlinie 455 möglicherweise nicht für Tests von Wechselwirkungen mit ISF bei Konzentrationen über 1 µM geeignet ist. Es wurde die Bedeutung des Verständnisses der Einschränkungen des Testsystems bei der Anwendung des Luciferase-Assays hervor und lieferten Einblicke in die Wechselwirkung zwischen ISF und dem Luciferase-Enzym.